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(51) International Patent Classification 5 : C07H 19/01, C07F 9/6533, 9/655 C07F 9/6527, C07K 7/56 C07F 9/117, 9/09, A61K 31/66 C12P 9/00, C12N 1/14 //(C12N 1/14, C12R 1/845)	A2	(11) International Publication Number: WO 92/06992 (43) International Publication Date: 30 April 1992 (30.04.92)																					
(21) International Application Number: PCT/US91/06816 (22) International Filing Date: 19 September 1991 (19.09.91) (30) Priority data: <table border="0"> <tr> <td>594,500</td> <td>9 October 1990 (09.10.90)</td> <td>US</td> </tr> <tr> <td>594,214</td> <td>9 October 1990 (09.10.90)</td> <td>US</td> </tr> <tr> <td>595,894</td> <td>11 October 1990 (11.10.90)</td> <td>US</td> </tr> <tr> <td>691,606</td> <td>26 April 1991 (26.04.91)</td> <td>US</td> </tr> <tr> <td>691,607</td> <td>26 April 1991 (26.04.91)</td> <td>US</td> </tr> <tr> <td>701,387</td> <td>16 May 1991 (16.05.91)</td> <td>US</td> </tr> <tr> <td>735,963</td> <td>25 July 1991 (25.07.91)</td> <td>US</td> </tr> </table> (60) Parent Application or Grant (63) Related by Continuation US 594,214 (CIP) Filed on 9 October 1990 (09.10.90) (71) Applicant (for all designated States except US): MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).		594,500	9 October 1990 (09.10.90)	US	594,214	9 October 1990 (09.10.90)	US	595,894	11 October 1990 (11.10.90)	US	691,606	26 April 1991 (26.04.91)	US	691,607	26 April 1991 (26.04.91)	US	701,387	16 May 1991 (16.05.91)	US	735,963	25 July 1991 (25.07.91)	US	(72) Inventors; and (75) Inventors/Applicants (for US only) : CHEN, Shieh-Shung, Tom [US/US]; 12 Scott Drive, Morganville, NJ 07751 (US). PETUCH, Brian, R. [US/US]; 41-2 Carriage Stop, Florence, NJ 08518 (US). HSU, Annjia, T. [US/US]; 4180 Randmore Court, Columbus, OH 43220 (US). ARISON, Byron, H. [US/US]; 88 Century Lane, Watchung, NJ 07060 (US). DUMONT, Francis [FR/US]; 54 West Cherry Street, Rahway, NJ 07065 (US). WHITE, Raymond, F. [US/US]; 12 Beckett Road, Englishtown, NJ 07726 (US). MATHRE, David, J. [US/US]; 19F Reading Road, Edison, NJ 08817 (US). WU, Jane, T. [US/US]; 5 Black Birch Road, Scotch Plains, NJ 07076 (US). SO, Lydia, T. [US/US]; 203 Prospect Avenue, Maywood, NJ 07607 (US). REAMER, Robert, A. [US/US]; 114 Maolis Avenue, Bloomfield, NY 07003 (US). (74) Agents: NORTH, Robert, J. et al.; Merck & Co., Inc., 126 East Lincoln Avenue, Rahway, NJ 07065-0907 (US). (81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US. Published <i>Without international search report and to be republished upon receipt of that report.</i>
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(54) Title: NEW PROCESS FOR BIOPHOSPHORYLATING ORGANIC COMPOUNDS

(57) Abstract

Described is a new microbial biophosphorylation process for regiospecifically phosphorylating the hydroxyl group, in a "phosphate active" hydroxyl containing organic compound, under biotransformation conditions utilizing the microorganism, *Rhizopus oryzae* ATCC No. 11145. The phosphorylated compounds produced by the process include e.g., macrolide FK-506 type immunosuppressants which are useful in preventing human host rejection of foreign organ transplants, e.g. bone marrow, liver, kidney, lung and heart transplants. Other hydroxyl containing compounds, applicable in this process include rapamycin, echinocandins, HIV protease inhibitors, simvastatin and zearalenone.

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⁺ Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

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TITLE OF THE INVENTION

NEW PROCESS FOR BIOPHOSPHORYLATING ORGANIC COMPOUNDS

15 CROSS REFERENCE TO RELATED APPLICATIONS

The present application is a combined continuation-in-part application of SN 594,500 (Case 18238) filed October 9, 1990; SN 594,214 (Case 18240) filed October 9, 1990; SN 595,894 (Case 18208) filed
20 October 11, 1990; SN 691,606 (Case 18261) filed April 26, 1991; SN 691,607 (Case 18382) filed April 26, 1991; SN 701,387 (Case 18340) filed May 16, 1991; and SN 735,963 (Case 18462) filed July 25, 1991.

25

BACKGROUND OF THE INVENTION1. Field of the Invention

This invention relates to a new regio-specific biophosphorylation process for producing
30 phosphorylated derivatives of "phosphate active" hydroxyl containing organic compounds utilizing the

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microorganism Rhizopus oryzae, ATCC No. 11145. The process involves contacting the microorganism and an organic compound containing a free phosphate active hydroxyl group, under biotransformation conditions, which phosphorylate the hydroxyl group. The process involves either resting Rhizopus cells or culturing the microorganism in the presence of the organic compound.

Rhizopus oryzae ATCC No. 11145 is known in the art primarily as a hydroxylating agent. It also degrades insect moulting hormones (J.C.S. Chem. Comm. 1974: 656-657, 1974); participates in hydroxylation of steroids (Can. J. Chem. 57:436-440 and 1585-1587, 1979; *ibid.*, 59: 1651-1655, 1981; *ibid.*, 63: 1127-1131, 1985; H.J. Peppier, ed., Microbial Technology Reinhold, New York, p. 288-297, 1967; U.S. Patent 2,646,370); is involved with transformations of sesquiterpene lactone costunolide (J.C.S, Perkin 1: 3022-3028, 1979); produces 16-hydroxyverrucarin A and B and 3'-hydroxyverrucarin A by transformation of verrucarins A and B (Appl. Environ, Microbiol, 46:480-483, 1983); and produces OH-products of imipramine (J. Pharmaceut. Sci. 70: 151-153, 1981). However, in the above references, there is no description of its ability as a regiospecific hydroxyl phosphorylating agent.

Rhizopus is a genus of fungi which commonly occurs on mature fruits, grain and vegetables, as well as soil. Typically they are saprobes and facultative parasites, and form a branched, aseptate mycelium. Rhizopus spp are used commercially, e.g., in the preparation of carboxylic acids or steroids, or in the metabolism of hydrocarbons.

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Culture or fermentation of Rhizopus arrhizus ATCC 11145 is straightforward, conventional and well known. See, for example, U.S. 4,410,629. Adaptations, modifications and variations of the protocol are given in the Examples are within the routine skill of the fermentation microbiologist.

Regiospecific biophosphorylating agents are desired in the art since standard chemical phosphorylation using, e.g., POCl_3 or PCl_5 , are generally non-regiospecific and do not produce selectively phosphorylated compounds, which can lead to improved water solubility and pharmacokinetics.

ATCC DEPOSIT

A sample of MF 4974 has been deposited at the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville MD 20852. The culture access designation is ATCC No. 11145.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is an ^1H nuclear magnetic resonance (NMR) spectrum taken at 400 MHz of C-32 phosphorylated FR-900520 in CDCl_3 .

Figure 2 exhibits the assigned molecular structure for C-32 phosphorylated FR-900520.

Figure 3 is ^1H nuclear magnetic resonance (NMR) spectrum taken at 400 MHz of the C-43 methylated phosphate ester of the phosphorylated rapamycin macrolide in MeOH.

Figure 4 exhibits the assigned molecular structure for the C-43 phosphorylated rapamycin macrolide.

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Figure 5 is ^1H nuclear magnetic resonance spectrum of echinocandin IIIA.

SUMMARY OF THE INVENTION

5 We have discovered a new overall process for selectively biophosphorylating an organic compound containing "phosphate active" hydroxyl groups. The organic compound is suspended in an aqueous medium with a strain of Rhizopus oryzae ATCC No. 11145 for a
10 sufficient time to produce the phosphorylated organic compound.

By the term "phosphate active hydroxyl containing organic compound" as used herein is meant a compound containing a hydroxyl group which can stereo-
15 specifically interact with the Rhizopus microorganism to undergo phosphorylation under biotransformation conditions. A simple test with the organic compound under the biotransformation conditions described herein will determine, without undue experimentation,
20 if the hydroxyl group in the organic molecule is phosphate active.

The process involves contacting resting Rhizopus cells in a phosphate buffered medium containing e.g. glycerol, as a carbon nutrient, or by
25 the fermentation of the microorganism Rhizopus oryzae, ATCC No. 11145 together, in the presence of the hydroxyl containing organic compound, e.g., an FK-506 type macrolide immunosuppressant i.e. FK-520, under submerged aerobic conditions in an aqueous
30 carbohydrate medium, containing a nitrogen nutrient, said conditions being conducted at a pH of about 7 for a sufficient time, e.g. 24 hours at 27°C, to selectively biophosphorylate the phosphate active

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hydroxyl group. Either process can be utilized but preferred is the process using resting cells.

It will be recognized by those skilled in the art that phosphorylation by Rhizopus oryzae ATCC 11145 is not limited to that particular strain. Rather, other Rhizopus oryzae strains can also be expected to be capable of acting to perform hydroxyl phosphorylation of hydroxy containing organic compounds.

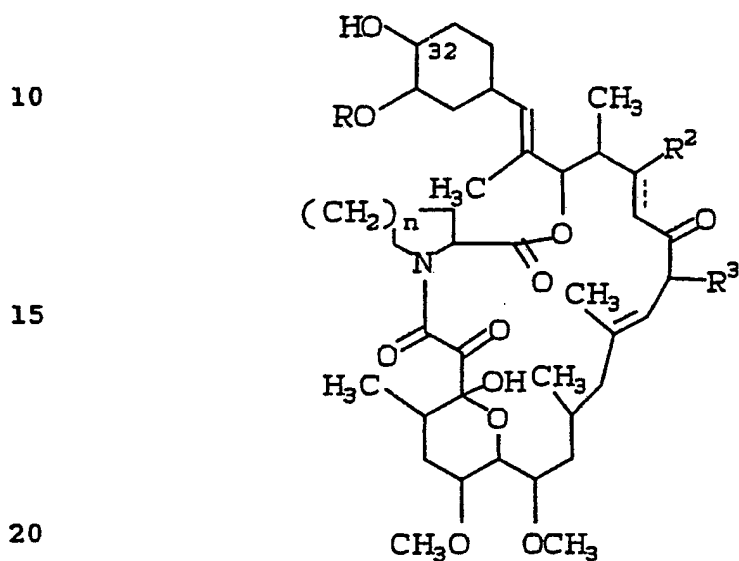
DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

In accordance with this invention there is provided a process for producing a biophosphorylated hydroxyl containing organic compound, wherein said hydroxyl group is phosphate reactive, comprising the step of contacting a strain of Rhizopus oryzae ATCC No. 11145 microorganism together with the hydroxyl containing organic compound in an aqueous medium containing a carbon nutrient at ambient temperature for a sufficient time to produce the biophosphorylated hydroxyl containing organic compound.

The present invention involves a biotransformation process, which involves the contacting of resting cells, or the fermentation of, the microorganism, Rhizopus oryzae, together with an organic compound containing a free hydroxy group, to produce the phosphorylated derivative. The microorganism is currently on deposit with the American Type Culture Collection, 12301 Parklawn Drive in Rockville, Maryland as ATCC No. 11145.

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The scope of the compounds included within the "phosphate reactive hydroxyl containing organic compounds" includes a C-32 hydroxy-containing macro-
 lide as described in Fujisawa's USP 4,894,366 of the
 5 formula:



25 wherein

R is H, C₁-C₄ alkyl,

R² is hydrogen, hydroxy or lower alkanoyloxy,

R³ is methyl, ethyl, propyl or allyl,

n is an integer of 1 or 2, and the symbol of a line

30 and dotted line is a single bond or a double bond,

and a pharmaceutically acceptable basic salt thereof.

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Specifically included is where the compound FK-506 is where R is methyl, R² is hydroxy, R³ is allyl, n is 2 and the double bond is absent; and the compound FK-520 is where R is methyl, R² is hydroxy, R³ is ethyl, n is 2 and the double bond is absent.

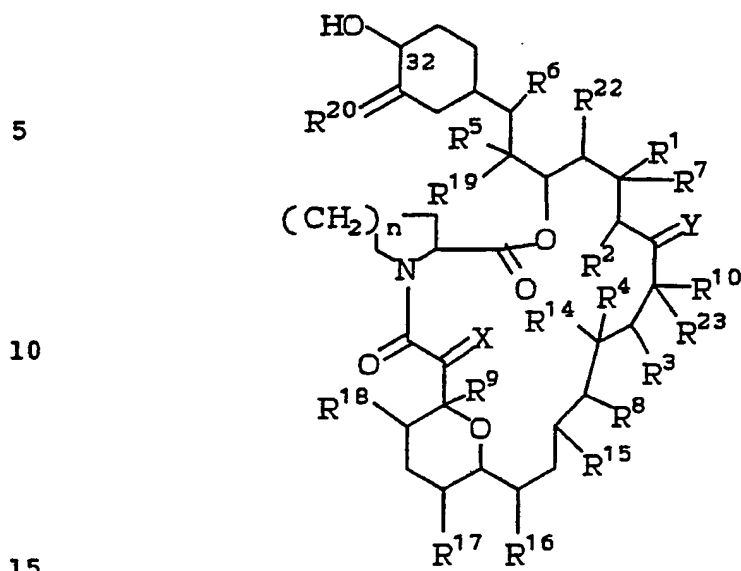
Also included are phosphate reactive hydroxyl containing compounds including the following FK-506 type compounds (from USP 4,894,366 to Fujisawa):

- 10 7-Allyl-1,14-dihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo-[22.3.1.0^{4,9}]octacos-18-ene-2,3,10,16-tetraone,
- 15 1,14-Dihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-23,25-dimethoxy-13,19,17,21,27-pentamethyl-11,28-dioxa-4-azatricyclo[22.3.1.0^{4,9}]octacos-18-ene-2,3,10,16-tetraone,
- 20 16-Allyl-1,13-dihydroxy-11-[-2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-22,24-dimethoxy-12,18,20,26-tetramethyl-10,27-dioxa-4-azatricyclo-[21.3.1.0^{4,8}]heptacos-17-ene-2,3,9,15-tetraone.

25 Further included within the term "FK-506 type macrolide" as used herein are specifically the compounds disclosed in Fison's EP0 0 323 042 of the formula:

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II

wherein each vicinal pair of substituents [R^1 and R^2], [R^3 and R^4], [R^5 and R^6] independently:

- a) represent two vicinal hydrogen atoms, or
- b) form a second bond between the vicinal carbon atoms to which they are attached;

in addition to its significance above,

R^2 can represent an C_1 - C_{10} alkyl group;

R^7 represents H, OH or O - C_1 - C_{10} alkyl, or in conjunction with R^1 it may represent $=O$;

R^8 and R^9 independently represent H or OH;

R^{10} represents H; C_1 - C_{10} alkyl, wherein said alkyl

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can be substituted by one or more hydroxyl groups;
C₁-C₁₀ alkenyl, which can be substituted by one or
more hydroxyl groups, or C₁-C₁₀ alkyl substituted by
=O;

- 5 X represents O, (H, OH), (H,H) or -CH₂O-;
Y represents O, (H, OH), (H,H), N-NR¹¹R¹² or N-OR¹³
wherein, R¹¹ and R¹² independently represent H,
C₁-C₁₀alkyl, C₁-C₁₀aryl or tosyl, and
R¹³, R¹⁴, R¹⁵, R¹⁶, R¹⁷, R¹⁸, R¹⁹, R²² and R²³
10 independently represent H or C₁-C₁₀ alkyl; R²⁰
independently represents O, or it can independently
represent (R²⁰_a,H); wherein R²⁰_a independently
represents OH, O-C₁-C₁₀ alkyl or OCH₂OCH₂CH₂OCH₃;
n is 1,2, or 3;
15 in addition to their significances above, Y, R¹⁰ and
R²³, together with the carbon atoms to which they are
attached, can represent a 5- or 6- membered N-, S- O-
containing heterocyclic ring, which is saturated or
unsaturated, and which can be substituted by one or
20 more groups selected from C₁-C₁₀ alkyl, hydroxyl,
C₁-C₁₀ alkyl substituted by one or more hydroxyl
groups, O-C₁-C₁₀ alkyl, benzyl and -CH₂Se(C₆H₅);
provided that when X and Y both represent O; R⁹
represents OH; R¹⁴, R¹⁵, R¹⁶, R¹⁷, R¹⁸, R¹⁹ and R²²
25 each represent methyl; R²⁰_a represents OCH₃; R⁸ and
R²³ each represent H; [R³ and R⁴] and [R⁵ and R⁶]
each represent a carbon-carbon bond;
and pharmaceutically acceptable salts thereof, which
includes acid addition salts of any amine groups
30 present.

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Preferably when R^2 , R^7 , R^{11} , R^{12} , R^{13} , R^{14} , R^{15} , R^{16} , R^{17} , R^{18} , R^{19} , R^{20a} , R^{22} and R^{23} comprise carbon-containing groups, those groups contain up to 10 carbon atoms, more preferably from 1 to 6, e.g., methyl or methoxyl.

Also preferred is wherein each of R^{14} , R^{15} , R^{16} , R^{17} , R^{18} , R^{19} and R^{22} represents methyl.

The alkyl groups: R^2 , R^7 , R^{11} , R^{12} , R^{13} , R^{14} , R^{15} , R^{16} , R^{17} , R^{18} , R^{19} , R^{20a} , R^{22} and R^{23} can comprise straight chain, branched and cyclic groups.

Alkyl groups substituted by =O, include R^{10} which can represent include ketone and aldehyde groups.

Preferably, R^{10} is allyl (i.e., prop-2-enyl), propyl ethyl or methyl.

Preferably, n is 2;

R^7 is H or OH;

R^1 and R^2 both represent H;

X is preferably O or (H, OH);

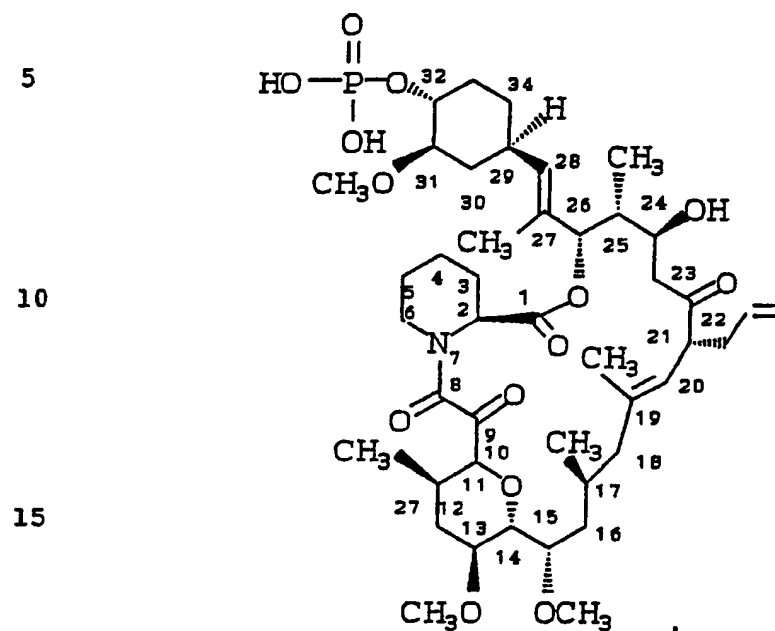
R^{20a} represents OH or OCH_3 ; and

when Y, R^{10} and R^{23} together represent a N-, S- or O- containing heterocyclic ring, preferred is where that ring is five-membered, more preferably a pyrrole or tetrahydrofuran ring.

Preferred embodiments as products are C-32 phosphorylated FK-506,

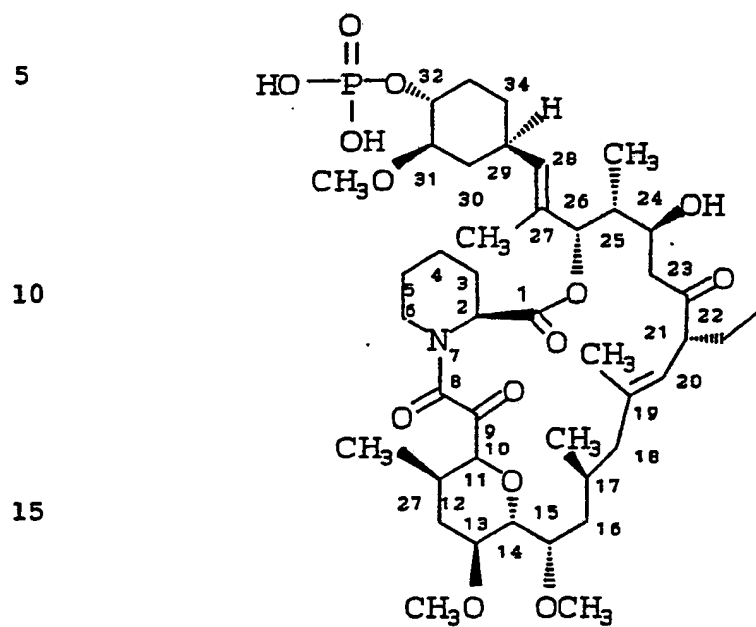
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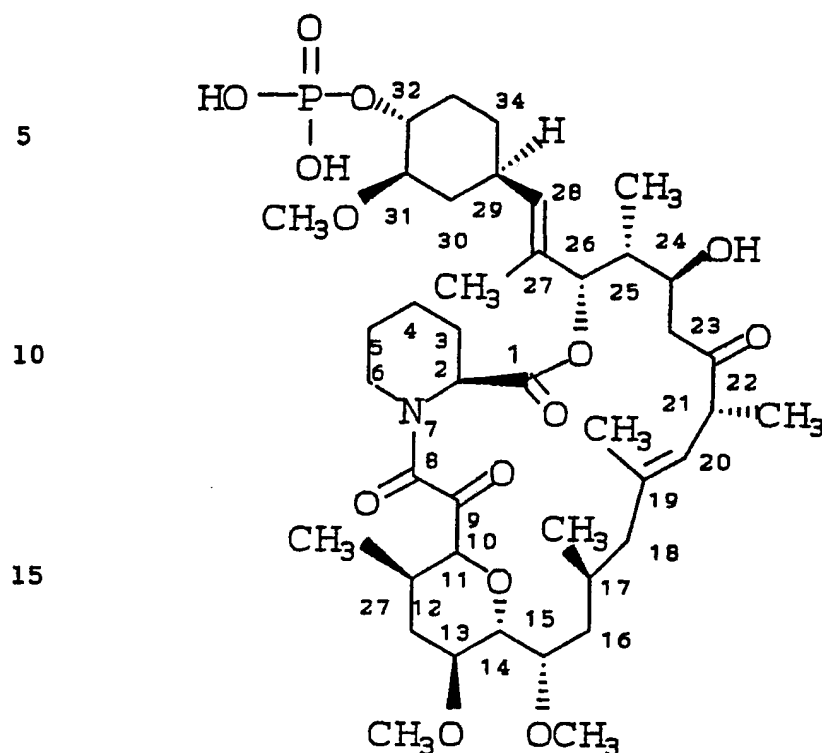
and C-32 phosphorylated FK-520,

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and C-32 phosphorylated FK-523;

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and the corresponding C-32 phosphorylated C-31 desmethyl analogs, and also the following specific compounds from EPO 323,042 to Fisons:

1,14-Dihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-18-[(phenylseleno)methyl]-16,26,28-trimethoxy-13,22,24,30-tetramethyl-11,17,31-trioxa-4-azatetracyclo[25.3.1.0^{4,9}.0^{16,20}]hentriacont-21-ene-2,3,10-trione,

17-Allyl-1,2,14-trihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo-[22.3.1.0^{4,9}]octacos-18-ene-3,10,16-trione,

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17-Allyl-1,2,14,16-tetrahydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo-[22.3.1.0^{4,9}]octacos-18-ene-3,10-dione,

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17-Propyl-1,14-dihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo-[22.3.1.0^{4,9}]octacosane-2,3,10,16-tetraone,

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17-Propyl-1,14-dihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylethyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo-[22.3.1.0^{4,9}]octacos-18-ene-2,3,10,16-tetraone,

15

17-Propyl-1,14-dihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylethyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo-[22.3.1.0^{4,9}]octacosane-2,3,10,16-tetraone,

20

17-Propyl-1-hydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo[22.3.1.0^{4,9}]-octacos-14,18-diene-2,3,10,16-tetraone,

25

17-Propyl-1-hydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo[22.3.1.0^{4,9}]-octacos]-18-ene-2,3,10,16-tetraone,

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- 17-Allyl-1,14,20-trihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo-[22.3.1.0^{4,9}]octacos-18-ene-2,3,10,16-tetraone,
5
- 17-(1-Hydroxyprop-2-enyl)-1,14,20-trihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo[22.3.1.0^{4,9}]octacos-18-ene-2,3,10,16-
10 tetraone,
- 17-Allyl-1,2-dihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo-
15 [22.3.1.0^{4,9}]octacos]-18-ene-3,10,16-trione,
- 17-Allyl-1,16-dihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo-
20 [22.3.1.0^{4,9}]octacos]-14,18-diene-2,3,10-trione,
- 17-Allyl-1-hydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo-
25 [22.3.1.0^{4,9}]octacos]-18-ene-2,3,10,16-tetraone,
- 17-(2,3-Dihydroxypropyl)-1,14-dihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo[22.3.1.0^{4,9}]octacos-18-ene-2,3,10,16-tetraone,
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- 17-Ethanalyl-1,14-dihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo-[22.3.1.0^{4,9}]octacos-18-ene-2,3,10,16-tetraone,
- 5
- 1,14-Dihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-26,28-dimethoxy-13,22,24,30-tetramethyl-11,31-dioxa-4,17-diazatetracyclo-[25.3.1.0^{4,9}.0^{16,20}]hentriaconta-16(20),18,21-triene-2,3,10-trione,
- 10
- 1,14-Dihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-26,28-dimethoxy-17-(2-hydroxyethyl)-13,22,24,30-tetramethyl-11,31-dioxa-4,17-diazatetracyclo[25.3.1.0^{4,9}.0^{16,20}]hentriaconta-16(20),18,21-triene-2,3,10-trione,
- 15
- 1,14-Dihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-26,28-dimethoxy-13,22,24,30-tetramethyl-17-phenylmethyl-11,31-dioxa-4,17-diazatetracyclo-[25.3.1.0^{4,9}.0^{16,20}]hentriaconta-16(20),18,21-triene-2,3,10-trione,
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- 1,14-Dihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylvinyl]-26,28-dimethoxy-13,22,24,30-tetramethyl-17-phenylmethyl-11,31-dioxa-4,17-diazatetracyclo[25.3.1.0^{4,9}.0^{16,20}]hentriaconta-16(20),18,21-triene-2,3,10-trione,
- 25

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17-Allyl-1,14-dihydroxy-12-[2-(4-hydroxy-3-methoxy-
cyclohexyl)-1-methylvinyl]-23,25-dimethoxy-
13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo-
[22.3.1.0^{4,9}]octacos-18-ene-2,3,10,16-tetraone C16
5 oxime,

17-Allyl-1,14-dihydroxy-12-[2-(4-hydroxy-3-methoxy-
cyclohexyl)-1-methylvinyl]-23,25-dimethoxy-
13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo-
10 [22.3.1.0^{4,9}]octacos-18-ene-2,3,10,16-tetraone C16
oxime O-methyl ether,

17-Propyl-1-hydroxy-12-[2-(3,4-dihydroxycyclohexyl)-1-
methylvinyl]-23,25-dimethoxy-13,19,21,27-tetramethyl-
15 11,28-dioxa-4-azatricyclo[22.3.1.0^{4,9}]octacos-18-ene-
2,3,10,16-tetraone,

1,14-Dihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-
1-methylvinyl]-23,25-dimethoxy-13,19,21,27-tetra-
20 methyl-17-(2-oxopropyl)-11,28-dioxa-4-azatricyclo-
[22.3.1.0^{4,9}]octacos-18-ene-2,3,10,16-tetraone,

17-Allyl-1,14-dihydroxy-12-[2-(4-hydroxy-3-
methoxycyclohexyl)-1-methylvinyl]-23,25-dimethoxy-
25 13,19,21,27-tetramethyl-11,28-dioxa-4-aza-spiro-
[tricyclo[22.3.1.0^{4,9}]octacos-18-ene-2,2'-oxirane,-
3,10,16-trione,

17-Ethanalyl-1,2,14-trihydroxy-12-[2-(4-hydroxy-3-
30 methoxycyclohexyl)-1-methylvinyl]-23,25-dimethoxy-
13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo-
[22.3.1.0^{4,9}]octacos-18-ene-3,10,16-trione,

-18-

1,14-Dihydroxy-12-[2-(4-hydroxy-3-methoxycyclohexyl)-
1-methylvinyl]-26,28-dimethoxy-18-[(phenylseleno)-
methyl-13,22,24,30-tetramethyl-11,17,31-trioxa-4-aza-
tetracyclo[25.3.1.0.4,⁹0.16,20]hentriaconta-16(20),21-
5 diene-2,3,10-trione,

4-Methyl-[17-Allyl-1,14-dihydroxy-12-[2-(4-hydroxy-
3-methoxycyclohexyl-1-methylvinyl]-23,25-dimethoxy-
13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo-
10 [22.3.1.0^{4,9}]octacos-18-ene-2,3,10-trione-16-ylidene]-
hydrazide,

or a pharmaceutically acceptable salt of any one
thereof,

15 In general, the phosphorylated organic com-
pound can be produced by either contacting resting
cells of *Rhizopus*, in an aqueous buffered phosphate
medium containing a carbon nutrient at ambient
temperature, or by culturing (fermenting) the above-
20 described microorganism, *Rhizopus oryzae*, in the
presence of the hydroxy containing organic compound
in an aqueous nutrient medium containing sources of
assimilable carbon and nitrogen, preferably under
submerged aerobic conditions (e.g. shaking culture,
25 submerged culture, etc.). The aqueous medium is
preferably maintained at a pH of about 7 at the
initiation and termination (harvest) of the fermen-
tation process. A higher pH leads to substantial
and/or total loss of product. The desired pH may be
30 maintained by the use of a buffer such as morpholino-
ethanesulfonic acid (MES), morpholinopropanesulfonic
acid (MOPS), and the like, or by choice of nutrient
materials which inherently possess buffering

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properties, such as production media described hereinbelow.

The preferred sources of carbon in the nutrient medium are carbohydrates such as glucose, xylose, galactose, glycerin, starch, dextrin, and the like. Other sources which may be included are maltose, rhamnose, raffinose, arabinose, mannose, salicin, sodium succinate, and the like.

The preferred sources of nitrogen are yeast extract, meat extract, peptone, gluten meal, cotton-seed meal, soybean meal and other vegetable meals (partially or totally defatted), casein hydrolysates, soybean hydrolysates and yeast hydrolysates, corn steep liquor, dried yeast, wheat germ, feather meal, peanut powder, distiller's solubles, etc., as well as inorganic and organic nitrogen compounds such as ammonium salts (e.g. ammonium nitrate, ammonium sulfate, ammonium phosphate, etc.), urea, amino acids, and the like.

The carbon and nitrogen sources, though advantageously employed in combination, need not be used in their pure form, because less pure materials which contain traces of growth factors and considerable quantities of mineral nutrients, are also suitable for use. When desired, there may be added to the medium mineral salts such as sodium or calcium carbonate, sodium or potassium phosphate, sodium or potassium chloride, sodium or potassium iodide, magnesium salts, copper salts, cobalt salts, and the like. If necessary, especially when the culture medium foams seriously, a defoaming agent, such as liquid paraffin, fatty oil, plant oil, mineral oil or silicone may be added.

-20-

As one of the starting materials in the process, the FK-520 starting material can be obtained by the fermentation of S. hygrosopicus var. ascomyceticus, ATCC No. 14891, as described in U.S. Patent 3,244,592, and by the fermentation of S. hygrosopicus subsp. yakushimaensis No. 7278, (to produce FR-900520, or "FK-520", and the other FK-506 type macrolides can be obtained by the processes) as described in EP0 Publication No. 0184162 to Fujisawa, and PCT WO 89/05304 to Fisons, said above references hereby incorporated by reference for this particular purpose.

As to the conditions for the production of phosphorylated organic compound in massive amounts, submerged aerobic cultural conditions are preferred therefor. For the production in small amounts, a shaking or surface culture in a flask or bottle is employed. Furthermore, when the growth is carried out in large tanks, it is preferable to use the vegetative form of the organism for inoculation in the production tanks in order to avoid growth lag in the process of production. Accordingly, it is desirable first to produce a vegetative inoculum of the organism by inoculating a relatively small quantity of culture medium with spores or mycelia of the organism produced in a "slant" and culturing said inoculated medium, also called the "seed medium", and then to transfer the cultured vegetative inoculum aseptically to large tanks. The fermentation medium, in which the inoculum is produced, is substantially the same as or different from the medium utilized for the production of the phosphorylated organic compound and is generally autoclaved to sterilize the medium

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prior to inoculation. The pH of the medium is generally adjusted to about 7.0 prior to the autoclaving step by suitable addition of an acid or base, preferably in the form of a buffering solution.

5 Agitation and aeration of the culture mixture may be accomplished in a variety of ways. Agitation may be provided by a propeller or similar mechanical agitation equipment, by revolving or shaking the fermentor, by various pumping equipment
10 or by the passage of sterile air through the medium. Aeration may be effected by passing sterile air through the fermentation mixture.

The fermentation is usually conducted at a temperature between about 20°C and 40°C, preferably
15 25-35°C, for a period of about 10 hours to 24 hours, which may be varied according to fermentation conditions and scales. Preferably, the production cultures are incubated for about 24 hours at 27°C on a rotary shaker operating at 220 rpm, wherein
20 the pH of the fermentation medium is maintained at 7.0 to harvest.

Preferred culturing/production media for carrying out the fermentation include the following media:

25

<u>Seed and Transformation Medium</u>		<u>g/L</u>
Glucose	20.0	
Soy Flour (Sigma)		5.0
Yeast Extract (Fidco)		5.0
NaCl	5.0	
K ₂ HPO ₄	5.0	
Adjust pH to 7.0		

30

-22-

The produced phosphorylated organic compound can be recovered from the culture medium by conventional means which are commonly used for the recovery of other known biologically active substances. The phosphorylated substance produced is found in the cultured mycelium and filtrate, and accordingly can be isolated and purified from the mycelium and the filtrate, which are obtained by filtering or centrifuging the cultured broth, by a conventional method such as concentration under reduced pressure, lyophilization, extraction with a conventional solvent, such as methanol and the like, pH adjustment, treatment with a conventional resin (e.g. anion or cation exchange resin, non-ionic adsorption resin, etc.), treatment with a conventional adsorbent (e.g. activated charcoal, silicic acid, silica gel, cellulose, alumina, etc.), crystallization, recrystallization, and the like. A preferred method is solvent extraction, particularly using methanol.

The phosphorylated organic compound obtained according to the resting cell or fermentation processes as explained above can be isolated and purified in a conventional manner, for example, extraction, precipitation, fractional crystallization, recrystallization, chromatography, and the like.

Suitable formulations of the material may also include conventional pharmaceutically acceptable biolabile esters of phosphorylated organic compound, formed via the hydroxy groups on the molecule, such as the acetate.

The phosphorylated organic compound, and particularly that of FK-520, of the present invention

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are water soluble possesses pharmacological activity such as immunosuppressive activity, antimicrobial activity, and the like, and therefore are useful for the treatment and prevention of the transplantation rejection of organs or tissues such as heart, kidney, liver, medulla ossium, skin, etc., graft-versus-host diseases by medulla ossium transplantation, autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, Hashimoto's thyroiditis, multiple sclerosis, myasthenia gravis, type I diabetes, uveitis, and the like.

The pharmaceutical composition of this invention can be used in the form of a pharmaceutical preparation, for example, in solid, semisolid or liquid form, which contains the instant invention compounds, as an active ingredient, in admixture with an organic or inorganic carrier or excipient suitable for external, enteral or parenteral applications. The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, and any other form suitable for use. The carriers which can be used are water, glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea and other carriers suitable for use in manufacturing preparations, in solid, semisolid, or liquid form, and in addition auxiliary, stabilizing, thickening and coloring agents and perfumes may be used. The active object compound is included in the pharmaceutical composition in an

-24-

amount sufficient to produce the desired effect upon the process or condition of diseases.

For applying this composition to a human, it is preferable to apply if by parenteral or enteral administration. While the dosage of therapeutically effective amount of the C-32 phosphorylated FK-506, varies from, and also depends upon the age and condition of each individual patient to be treated, a daily dose (calculated on the basis of a 70 kg man) of about 0.01-1000 mg, preferably 0.1-500 mg and more preferably 0.5-100 mg, of the active ingredient is generally given for treating diseases, and an average single dose of about 0.5 mg, 1 mg, 5 mg, 10 mg, 50 mg, 100 mg, 250 mg and 500 mg is generally administered.

Rapamycin

It has been further found that a new immunosuppressant, a phosphorylated rapamycin macrolide, can be obtained via the present process by contacted resting Rhizopus cells in a phosphate buffered medium containing glycerol as a carbon nutrient, or by the fermentation of the microorganism Rhizopus oryzae, ATCC No. 11145 together, in the presence of the macrolide rapamycin, under submerged aerobic conditions in an aqueous carbohydrate medium, containing a nitrogen nutrient, said conditions being conducted at a pH of about 7 for a sufficient time, e.g. 24 hours at 27°C, to selectively C-43 phosphorylate the rapamycin type macrolide. Either process can be utilized but preferred is the process using resting cells.

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The resultant C-43 phosphorylated macrolide exhibits immunosuppressant activity, similar to rapamycin i.e., inhibition of T-cell proliferation of mouse T lymphocytes stimulated with the combination of interleukin-2 plus PMA. A positive sample in this assay will inhibit T-cell proliferation, as indicated by reduced tritiated thymidine uptake.

Also, in accordance with this invention, there is provided a process for producing an immunosuppressant, identified as a phosphorylated macrolide comprising the step of contacting a strain of a Rhizopus microorganism capable of phosphorylating free hydroxy groups, e.g., Rhizopus oryzae, and specifically Rhizopus oryzae ATCC No. 11145, together with rapamycin macrolide (See U.S. Patent 3,929,992 for its preparation), in an aqueous medium, containing a carbon nutrient, preferably at a pH below about 8.0, for a sufficient time to produce the C-43 phosphorylated macrolide.

Also provided is the unfiltered broth produced by the above process, which exhibits positive inhibition of T-cell activation.

Also provided is a new immunosuppressant being a phosphorylated macrolide which exhibits a proton nuclear magnetic resonance spectrum as identified in Figure 3, and a molecular weight of 993 as obtained by (FAB) mass spectrometry.

Also provided is a pharmaceutical composition for the treatment of immunoregulatory disorders and diseases containing a therapeutically effective amount of the phosphorylated macrolide in combination with a pharmaceutically acceptable, substantially non-toxic carrier or excipient.

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In addition, there is provided a method of use for treating a human host to prevent organ transplantation rejection, i.e., heart, kidney, liver, lung, bone marrow, or for treating autoimmune diseases i.e., juvenile diabetes mellitus comprising administering to said host a therapeutically effective amount of the phosphorylated macrolide.

The present invention involves a biotransformation process, which involves the contacting of resting cells, or the fermentation of, the micro-organism, Rhizopus oryzae, together with a rapamycin macrolide containing a free hydroxy group, to produce the phosphorylated macrolide derivative. The micro-organism is currently on deposit with the American Type Culture Collection, 12301 Parklawn Drive in Rockville, Maryland as ATCC No. 11145.

By the term "phosphorylated macrolide" as used herein is meant the compound having a proton NMR spectrum as illustrated in Figure 3 (of the methylated derivative), a mass spectrum molecular ion of 993, and an assigned molecular structure of the formula:

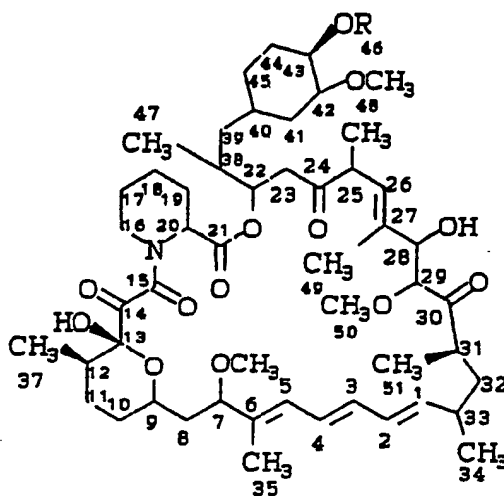
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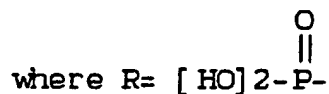
-27-

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In general, the C-43 phosphorylated macrolide can be produced by either contacting resting cells, of *Rhizopus* in an aqueous buffered phosphate medium containing a carbon nutrient at ambient temperature, or by culturing (fermenting) the above-described microorganism, *Rhizopus oryzae*, in the presence of the known rapamycin macrolide, described in U.S. Patent 3,929,992, in an aqueous nutrient medium containing sources of assimilable carbon and nitrogen, preferably under submerged aerobic conditions (e.g. shaking culture, submerged culture, etc.). The aqueous medium is preferably maintained at a pH of about 7 at the initiation and termination (harvest) of the fermentation

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process. A higher pH leads to substantial and/or total loss of product. The desired pH may be maintained by the use of a buffer such as morpholinoethanesulfonic acid (MES), morpholino-
5 propanesulfonic acid (MOPS), and the like, or by choice of nutrient materials which inherently possess buffering properties, such as production media described hereinbelow.

The preferred sources of carbon in the
10 nutrient medium are carbohydrates such as glucose, xylose, galactose, glycerin, starch, dextrin, and the like. Other sources which may be included are maltose, rhamnose, raffinose, arabinose, mannose, salicin, sodium succinate, and the like.

15 The preferred sources of nitrogen are yeast extract, meat extract, peptone, gluten meal, cottonseed meal, soybean meal and other vegetable meals (partially or totally defatted), casein hydrolysates, soybean hydrolysates and yeast hydrolysates, corn
20 steep liquor, dried yeast, wheat germ, feather meal, peanut powder, distiller's solubles, etc., as well as inorganic and organic nitrogen compounds such as ammonium salts (e.g. ammonium nitrate, ammonium sulfate, ammonium phosphate, etc.), urea, amino
25 acids, and the like.

The carbon and nitrogen sources, though advantageously employed in combination, need not be used in their pure form, because less pure materials which contain traces of growth factors and consider-
30 able quantities of mineral nutrients, are also suitable for use. When desired, there may be added to the medium mineral salts such as sodium or calcium

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carbonate, sodium or potassium phosphate, sodium or potassium chloride, sodium or potassium iodide, magnesium salts, copper salts, cobalt salts, and the like. If necessary, especially when the culture
5 medium foams seriously, a defoaming agent, such as liquid paraffin, fatty oil, plant oil, mineral oil or silicone may be added.

The rapamycin starting material can be obtained by the known fermentation of S. hygroscopicus,
10 NRRL No. 5491, as described in U.S. Patent 3,929,992.

As to the conditions for the production of the phosphorylated macrolide in massive amounts, submerged aerobic cultural conditions are preferred therefor. For the production in small amounts, a
15 shaking or surface culture in a flask or bottle is employed. Furthermore, when the growth is carried out in large tanks, it is preferable to use the vegetative form of the organism for inoculation in the production tanks in order to avoid growth
20 lag in the process of production. Accordingly, it is desirable first to produce a vegetative inoculum of the organism by inoculating a relatively small quantity of culture medium with spores or mycelia of the organism produced in a "slant" and culturing said
25 inoculated medium, also called the "seed medium", and then to transfer the cultured vegetative inoculum aseptically to large tanks. The fermentation medium, in which the inoculum is produced, is substantially the same as or different from the medium utilized for
30 the production of the phosphorylated macrolide and is generally autoclaved to sterilize the medium prior to inoculation. The pH of the medium is generally

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adjusted to about 7.0 prior to the autoclaving step by suitable addition of an acid or base, preferably in the form of a buffering solution.

Agitation and aeration of the culture mixture may be accomplished in a variety of ways. Agitation may be provided by a propeller or similar mechanical agitation equipment, by revolving or shaking the fermentor, by various pumping equipment or by the passage of sterile air through the medium. Aeration may be effected by passing sterile air through the fermentation mixture.

The fermentation is usually conducted at a temperature between about 20°C and 40°C, preferably 25-35°C, for a period of about 10 hours to 24 hours, which may be varied according to fermentation conditions and scales. Preferably, the production cultures are incubated for about 24 hours at 27°C on a rotary shaker operating at 220 rpm, wherein the pH of the fermentation medium is maintained at 7.0 to harvest.

Preferred culturing/production media for carrying out the fermentation include the following media:

25	<u>Seed and Transformation Medium</u>	<u>g/L</u>
	Dextrose	20.0
	Soy Meal (Sigma)	5.0
	Yeast Extract (Fidco)	5.0
	NaCl	5.0
30	K ₂ HPO ₄	5.0
	Adjust pH to 7.0	

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The produced phosphorylated macrolide can be recovered from the culture medium by conventional means which are commonly used for the recovery of other known biologically active substances. The phosphorylated macrolide produced is found in the cultured mycelium and filtrate, and accordingly can be isolated and purified from the mycelium and the filtrate, which are obtained by filtering or centrifuging the cultured broth, by a conventional method such as concentration under reduced pressure, lyophilization, extraction with a conventional solvent, such as methanol and the like, pH adjustment, treatment with a conventional resin (e.g. anion or cation exchange resin, non-ionic adsorption resin, etc.), treatment with a conventional adsorbent (e.g. activated charcoal, silicic acid, silica gel, cellulose, alumina, etc.), crystallization, recrystallization, and the like. A preferred method is solvent extraction, particularly using methanol.

The product phosphorylated macrolide from the fermentation exhibits positive immunosuppressive activity by the "T-cell proliferation assay" and possesses utility on this basis and exhibits the following physical characteristics:

25

1. White amorphous powder
2. Solubility in methanol
3. Molecular weight of 993, as determined by FAB mass spectroscopy and is consistent with the assigned molecular structure in Figure 3.

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The phosphorylated macrolide obtained according to the resting cell or fermentation processes as explained above can be isolated and purified in a conventional manner, for example, extraction, precipitation, fractional crystallization, recrystallization, chromatography, and the like.

Suitable formulations of the material may also include conventional pharmaceutically acceptable biolabile esters of the phosphorylated macrolide, formed via the hydroxy groups on the molecule, such as the acetate.

It is to be noted that in the aforementioned fermentation reactions and the post-treatment of the fermentation mixture therein, the tautomeric and conformational isomer(s) of the phosphorylated macrolide, including those due to rearrangement of the hemiketal ring system are also included within the scope of the present invention.

The phosphorylated macrolide of the present invention possesses pharmacological activity such as immunosuppressive activity, antimicrobial activity, and the like, and therefore are useful for the treatment and prevention of the transplantation rejection of organs or tissues such as heart, kidney, liver, medulla ossium, skin, etc., graft-versus-host diseases by medulla ossium transplantation, autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, Hashimoto's thyroiditis, multiple sclerosis, myasthenia gravis, type I diabetes, uveitis, and the like.

The pharmaceutical composition of this invention can be used in the form of a pharmaceutical

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preparation, for example, in solid, semisolid or liquid form, which contains the instant invention compounds, as an active ingredient, in admixture with an organic or inorganic carrier or excipient suitable for external, enteral or parenteral applications. The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, and any other form suitable for use. The carriers which can be used are water, glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea and other carriers suitable for use in manufacturing preparations, in solid, semisolid, or liquid form, and in addition auxiliary, stabilizing, thickening and coloring agents and perfumes may be used. The active object compound is included in the pharmaceutical composition in an amount sufficient to produce the desired effect upon the process or condition of diseases.

For applying this composition to a human, it is preferable to apply it by parenteral or enteral administration. While the dosage of therapeutically effective amount of the phosphorylated macrolide, varies from, and also depends upon the age and condition of each individual patient to be treated, a daily dose (calculated on the basis of a 70 kg man) of about 0.01-1000 mg, preferably 0.1-500 mg and more preferably 0.5-100 mg, of the active ingredient is generally given for treating diseases, and an average single dose of about 0.5 mg, 1 mg, 5 mg, 10 mg, 50

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mg, 100 mg, 250 mg and 500 mg is generally administered.

Echinocandins

5 The present invention process also is applicable in a process for selectively biophosphorylating a cyclic lipopeptide related to echinocandins and having a peptide skeleton bearing several hydroxy groups wherein in said phosphorylated
10 cyclic lipopeptide, the phosphate group is attached to the hydroxy group of the 4-hydroxyproline component of the lipopeptide.

Echinocandins or echinocandin compounds are cyclohexapeptide compounds having a lipophilic side
15 chain and having antifungal properties. Many are natural products but many compounds are semi-synthetic. The natural products are described in the literature by such names as echinocandins, aculeacins, mulundocandin, by number designations or
20 by structure. Many have been known a long time and the structure and properties may be found summarized in the CRC Handbook of Antibiotic Compounds, Vol IV, Part I, pp 355-367, CRC Press, Inc., Boca Raton, Fla. 1980. Still others include a more recently
25 discovered compound such as that described in U.S. Patent 4,931,352.

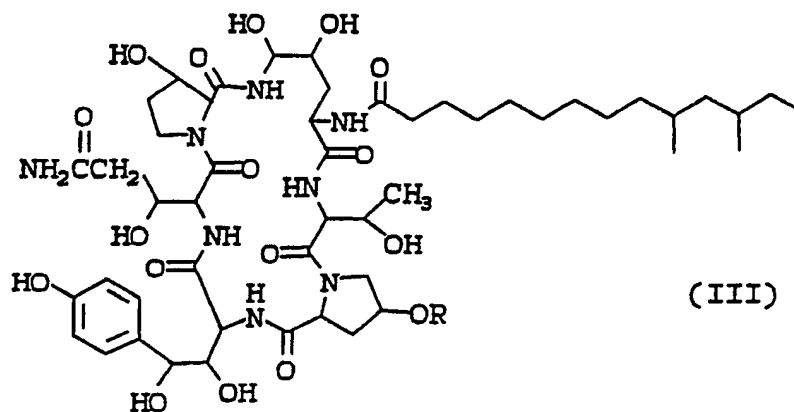
The present invention is especially directed to a compound having the formula (III):

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wherein R is $\text{-}\overset{\text{O}}{\overset{\parallel}{\text{P}}}(\text{OH})_2$ or a cation salt thereof.

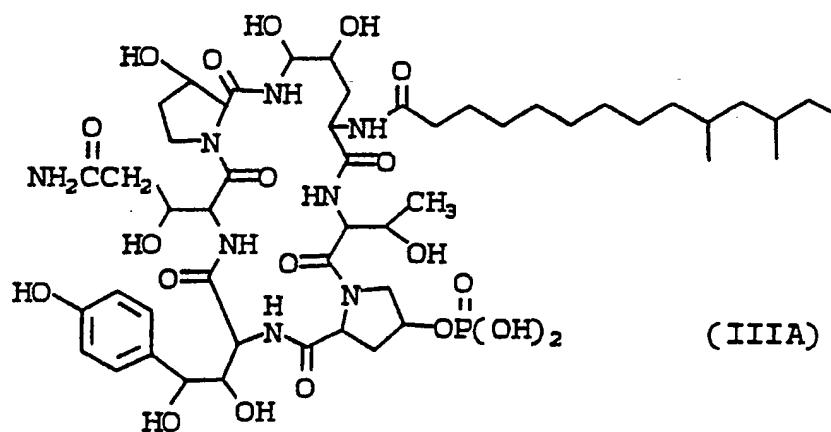
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By "cation salt" is meant a salt of Li, K, Mg, Na, Ca, and $(\text{C}_1\text{-C}_4 \text{ alkyl})\text{ammonium}$.

When R is $\text{-}\overset{\text{O}}{\overset{\parallel}{\text{P}}}(\text{OH})_2$, the compound may be represented by formula (IIIA), and hereinafter referred to as Compound IIIA.

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BRIEF DESCRIPTION OF THE DRAWING

Fig. 5 is nuclear magnetic resonance spectrum of Compound III in which R is $\begin{array}{c} \text{O} \\ \parallel \\ -\text{P}(\text{OH})(\text{OK}) \end{array}$.

5

MASS SPECTRAL DATA

Compound IIIA has a molecular weight of 1144 by FAB-MS (observed $(\text{M} + \text{Na})^+$ of 1167).

10 NMR DATA

^1H NMR Spectra of the compound isolated as a monopotassium salt in CD_3OD at 400 MHz is seen in Fig. 5; and

^{13}C NMR chemical shifts of the same isolate in CD_3OD at 100 MHz are as follows:
 δ_{C} 177.2, 175.9, 174.3, 173.2, 172.9, 172.58, 172.57, 169.0, 158.4, 133.1, 129.6, 116.2, 77.0, 75.8, 75.2 (doublet, $J_{\text{CP}}=4.0$ Hertz), 74.3, 73.9, 70.6, 70.5, 69.7, 68.3, 62.7, 58.3, 56.3, 56.1, 55.6, 51.2, 47.0, 45.9, 39.4, 38.1, 37.6, 36.7, 35.0, 34.6, 32.9, 31.24, 31.20, 30.8, 30.6, 30.3, 28.1, 27.0, 20.7, 20.2, 19.8, 11.6.

On the basis of these and other data, Compound IIIA is believed with considerable certainty to have the structure indicated.

Compound IIIA is a white solid soluble in water and polar solvents such as lower alkanols and in dilute alkali metal, magnesium, calcium, and tetra (lower alkyl)ammonium bases. From the bases, salts in which R is a cation salt of phosphate may be obtained.

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The compound of this invention has similar antibiotic properties as the non-phosphorylated compound and thus would be useful as an antibiotic for the control of parasites, especially Pneumocystis carinii, the causative agent of pneumocystis pneumonia, a particular problem with immune compromised patients, and for the control of fungi. Its antifungal properties are particularly useful against certain strains of yeast, such as Candida albicans and Candida tropicalis.

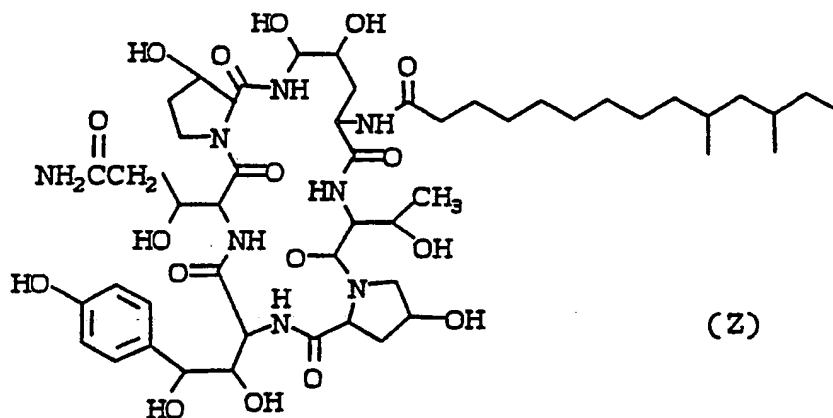
The compound in which R is $\overset{\text{O}}{\parallel}\text{-P(OH)}_2$ is conveniently produced by incubating a compound having the formula (Z) (Compound Z)

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with induced resting cells of Rhizopus arrhizus ATCC 11145 maintained in the Merck Culture Collection as MF 4974. The culture was originally obtained from the American Type Culture Collection at 12301
5 Parklawn Drive, Rockville, MD 20852.

Compound Z maybe produced by cultivating Zalarion arboricola ATCC 74030 in nutrient medium as hereinafter described as well as by methods described in copending applications S.N. 492,025, filed
10 March 12, 1990 and S.N. 492,026, also filed March 12, 1990.

The microorganism Rhizopus arrhizus ATCC 11145 is also known as Rhizopus oryzae (J.J. Ellis, 1985, Mycologia 77: 243-247). The species has also
15 been described under the names Rhizopus nodosus and Rhizopus tritici. The strain MF 4974, ATCC 11145, exhibits all the essential features of R. arrhizus described by M.A.A. Schipper under the name R. oryzae, CBS Studies in Mycology 25:1-19 (1984).
20 During a recent regrowth of MF4974 ATCC 11145, the following diagnostic characteristics were observed.

The strain is apparently heterothallic because zygosporangium formation was not observed. Colonies grow on most standard mycological media, but
25 on cornmeal agar (Difco) are extremely fast-growing, reaching 35 mm in diameter in 36 hours at 20°C, reaching >90 mm in 36 hours at 37°C, with sporangiophores completely filling Petri dishes, hyaline at first but soon becoming pale yellowish
30 gray to light gray, brownish gray, finally dark gray. Sporangioophores 200-1000 µm tall, 7.5-19 µm wide, aseptate, straight to curved at base, flared at

-39-

apex, sometimes furcate, with walls slightly thickened, with minutely granular surface, pale yellowish brown or yellowish gray, arising from rhizoidal hyphae. Rhizoidal hyphae consisting of
5 3-10, thick, often contorted branches.

Sporangia globose to subglobose, slightly flattened on underside, 100-230 μ m in diameter, opaque, with surface spiny, dark gray to black. Columellae 18-50 μ m in diameter, hemispherical
10 to subglobose, smooth, often collapsing, without adhering sporangia remnants. Sporangiospores 5-8 X 2.5-5 μ m, subglobose to irregularly elliptical, or angular in sideview, with faint to prominent longitudinal striations, hyaline to pale yellowish
15 brown.

Although the invention is discussed hereinbelow principally with respect to a specific strain, not only the strain described above, but varieties and mutants, whether obtained by natural
20 selection, produced by the action of various mutating agents such as ionizing radiation or chemical agents such as nitrosoguanidine are contemplated within the scope of the present invention.

Compound IIIA may be produced by cultivating
25 Rhizopus arrhizus ATCC 11145 in a suitable nutrient medium containing Compound Z under conditions hereinafter described and thereafter recovering from the product medium by extracting the desired product from the fermentation medium with a suitable solvent,
30 concentrating the component containing the desired compound, and then subjecting the concentrated material to chromatographic separation.

-40-

The cultivation is carried out in a medium containing sources of carbon and nitrogen assimilable by the microorganism.

The sources of carbon include glycerol, 5 sugars, sugar alcohols, starches and other carbohydrates, or carbohydrate derivatives such as dextran, cerelese, as well as complex nutrients such as oat flour, corn meal, millet, corn and the like. The exact quantity of the carbon source which is utilized 10 in the medium will depend, in part, upon the other ingredients in the medium, but it is usually found that an amount of carbohydrate between 0.5 and 40% by weight of the medium is satisfactory. These carbon sources can be used individually or several such 15 carbon sources may be combined in the same medium.

The sources of nitrogen include amino acids such as glycine, arginine, threonine, methionine and the like, ammonium salt, and complex sources such as yeast hydrolysates, yeast autolysates, yeast cells, 20 tomato paste, soybean meal, casein hydrolysates, yeast extracts, corn steep liquors, distillers solubles, cottonseed meal, meat extract, and the like. The various sources of nitrogen can be used alone or in combination in amounts ranging from 0.2 25 to 10 percent by weight of the medium.

In addition, the medium should contain a phosphate salt. The phosphate salt should be at least about 10 percent by weight of the solid components. It is preferably from about 12 to about 15 percent. 30 A particularly suitable medium is soy-glucose medium of the following composition which may be employed both as a seed medium and a culture medium:

-41-

	<u>Soy-Glucose Medium</u>	<u>g/l</u>
	Glucose	20.0
	Soya meal	5.0
	Fidco yeast extract*	5.0
5	NaCl	5.0
	K ₂ HPO ₄	5.0

Adjust pH to 5

10 *Fidco yeast extract is a nitrogen source,
 product of Difco Laboratories, Detroit MI.

 The fermentation may be carried out by
first preparing a seed culture. In preparing a seed
15 culture, spores of Rhizopus arrhizus are obtained
 from oatmeal agar slants of MF 4974 maintained in
 the Merck Culture Collection and dispersed in water
 to obtain a spore suspension containing about 7×10^9
 spores per milliliter.

20 The spore suspension of MF 4974 is
 inoculated into a seed flask containing the soy
 glucose broth and the inoculated suspension incubated
 on a rotary shaker in the temperature range of from
 about 15°C to about 30°C, preferably 25° to 28°C.
25 The agitation may be up to 400 rpm but generally
 about 220 rpm is preferred. The incubation is
 carried out over a period of at least 24 hours to
 about two days.

30 When growth is abundant, the mycelia are
 harvested by filtering through a nylon mesh. For
 biophosphorylation, the mycelia are suspended in a
 phosphate buffer containing 3 percent glycerol or
 some other simple carbon source. Compound Z then is

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added at a concentration of about 50 µg/ml in dimethylsulfoxide (DMSO). The pH of the production medium is important. The flasks are incubated, preferably with shaking at 220 rpm at 27°C for 24 to 48
5 hours to produce Compound IIIA. It is critical that it be maintained in the range of about 6.0 to 6.3.

After completion of the incubation period the contents of all the flasks are pooled and filtered through a nylon mesh filter. The mycelial
10 cake on the filter is slurried with aqueous methanol and filtered. The procedure is repeated with the filter cake and the filtrate loaded onto a styrene/divinylbenzene column. The column is then washed with water and the phosphorylated product eluted with
15 20 percent aqueous acetonitrile and the remaining metabolite and substrate eluted with 70 percent aqueous acetonitrile.

After elution, the fractions may be assayed by HPLC. The fractions determined to have the desired
20 product as indicated by a retention time of 12.8 mins. are combined and concentrated under reduced pressure to obtain the product as residue.

The salts, i.e., where R is a cationic salt of the phosphate, may be prepared by intimately
25 contacting a base corresponding to the cation in an alcoholic or other polar solvent, then concentrating to initiate crystallization of the salt. Thereafter, the salt is recovered by filtration.

One method of preparing salts is to apply an
30 aqueous solution of the acid onto a non-functionalized resin column. Representative resins include "AMBERCHROM"-161 (divinylbenzene/polystyrene resin,

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obtainable from TosoHaas; trademark name registered by Rohm and Haas), "DIAION" HP-20 and SP-207 (cross-linked styrene-divinylbenzene and brominated styrene-divinylbenzene, respectively, products of Mitsubishi Chemical). The column is then washed with aqueous MH_2PO_4 or $\text{M}'(\text{H}_2\text{PO}_4)_2$ where M and M' are monovalent and divalent cations respectively, thereby converting the acid to a mono-cation salt form. The column is washed with water to remove excess inorganic phosphate salt. The product M or M' salt is then removed from the column by applying an aqueous eluant having greater than 50 percent organic content. Useful eluants are 80 percent acetonitrile, 80 percent ethanol or 80 percent methanol. The product is isolated by concentration to dryness and/or lyophilization of the eluate.

This procedure also may be employed to prepare one salt from another.

Alternatively, the acid is dissolved in an aqueous mobile phase containing low amount of organic solvent such as acetonitrile and containing phosphate salt thereby forming a solution of the salt of the acid. The solution is subjected to reduced pressure to remove the acetonitrile, then applied to a C-18 extraction column to retain the salt of the product on the column. The salt of the product then may be removed as above described.

As previously noted, the phosphate is a compound which is active against certain yeast fungi such as *C. albicans* and *C. tropicalis*. The activity may be seen in a microbroth dilution assay employing a Yeast Nitrogen Base (Difco) with 1% dextrose

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(YNBD). In carrying out the assay, Compound IIIA was solubilized in 10 percent dimethyl sulfoxide (DMSO) and diluted to 2560 $\mu\text{g/ml}$. The compounds were further diluted to 256 $\mu\text{g/ml}$ in YNBD. Then 0.15 ml
5 of the suspension was dispensed to the first row of a 96-well plate (each well containing 0.15 ml of YNBD) resulting in a drug concentration of 128 $\mu\text{g/ml}$. Two-fold dilutions were then made to obtain final drug concentrations ranging from 128 to 0.06 $\mu\text{g/ml}$.

10 The yeast cultures, maintained on Sabouraud dextrose agar were transferred to YM broth (Difco) and incubated overnight at 35°C with shaking (250 rpm). After incubation, each culture was diluted in sterile water to yield a final concentration of $1-5 \times 10^6$ colony forming units (CFU)/ml.
15

96-well microplates were inoculated using a MIC-2000 (Dynatech) which delivers 1.5 μl per well yielding a final inoculum per well of $1.5-7.5 \times 10^3$ cells. The microplates were incubated at 35°C for 24
20 hours. The minimum inhibitory concentrations (MICs) were recorded as the lowest concentrations of drug showing no visible growth.

After recording the MIC, the plates were shaken to resuspend the cells. Thereafter, 1.5 μl
25 samples from the wells in the 96-well microplate were transferred to a single well tray containing Sabouraud dextrose agar. The inoculated trays were incubated 24 hours at 28°C and then read. The MFC is defined as the lowest concentration of drug showing no growth
30 or less than 4 colonies per spot.

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		Minimum Fungicidal Concentration ($\mu\text{g/ml}$)
<u>Fungus</u> <u>Strain No.</u>		<u>Compound IIIA</u>
<u>Candida albicans</u>		
5	MY 1055	64
	MY 1028	64
<u>Candida tropicalis</u>		
10	MY 1012	32

The foregoing illustrates particular suitability for treating mycotic infections.

The compounds of the present invention may be employed in inhibiting or alleviating Pneumocystis
15 carinii infections. In such use, Compound III/IIIA or a composition containing Compound III/IIIA may be administered in a therapeutically effective or inhibitory amount to subjects infected with or susceptible to being infected with Pneumocystis
20 carinii.

The suitability of the compounds of the present invention for therapeutic or anti-infective purposes may be determined in studies on immuno-suppressed rats when Sprague-Dawley rats (weighing
25 approximately 200 grams) are immunosuppressed with dexasone in the drinking water (2.0 mg/L) and maintained on a low protein diet for five weeks to induce the development of pneumocystis pneumonia from a latent infection. Before drug treatment, two rats
30 are sacrificed to confirm the presence of Pneumocystis carinii pneumonia (PCP). Six rats then are injected twice daily for four days intravenously (I.V.) via

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the tail vein with Compound III in 0.25 ml of vehicle (distilled water). A vehicle control is also carried out. All animals continue to receive dexasone in the drinking water and low protein diet during the treatment period. At the completion of the treatment, all animals are sacrificed, the lungs are removed and processed, and the extent of disease determined by microscopic analysis of stained slides.

A similar experiment may be carried out in which the rats are injected intraperitoneally (I.P.) twice daily for four days and then sacrificed, the lungs removed and processed, and the extent of disease determined by microscopic analysis of stained slides.

The outstanding properties are most effectively utilized when the compound is formulated into novel pharmaceutical compositions with a pharmaceutically acceptable carrier according to conventional pharmaceutical compounding techniques.

The novel compositions contain at least a therapeutic antifungal or antipneumocystis amount of the active compound. Generally, the composition contains at least 1% by weight of Compound III. Concentrate compositions suitable for dilutions prior to use may contain 90% or more by weight. The compositions include compositions suitable for rectal, topical, parenteral (including subcutaneous, intramuscular, and intravenous), pulmonary (nasal or buccal inhalation), nasal administration, or insufflation. The compositions may be prepacked by intimately mixing Compound III with the components suitable for the medium desired.

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When the compound is for antifungal use any method of administration may be used. For treating mycotic infection, oral administration is frequently preferred. When oral administration is to be
5 employed, it may be with a liquid composition or a solid composition. For liquid preparations, the therapeutic agent is preferably formulated with water or aqueous compositions, but if desired, may be formulated with glycols, oils, alcohols, and the
10 like. For solid preparations such as capsules and tablets, solid carriers such as starches, sugars, kaolin, ethyl cellulose, calcium and sodium carbonate, calcium phosphate, kaolin, talc, lactose, generally with lubricant such as calcium stearate, together
15 with binders, disintegrating agents and the like. Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage form. It is especially advantageous to formulate the compositions in unit dosage form (as
20 hereinafter defined) for ease of administration and uniformity of dosage. Composition in unit dosage form constitutes an aspect of the present invention.

The Compound III is preferably formulated in aqueous therapeutic compositions for intravenous or
25 intraperitoneal injection or aerosol when use against Pneumocystis carinii is contemplated, and may be presented in unit dosage form in ampoules or in multidose containers, if necessary with an added preservative. The compositions may also take such
30 forms as solutions in aqueous vehicles such as 0.85 percent sodium chloride or 5 percent dextrose in water, and may contain formulating agents such as

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stabilizing and/or dispersing agents. Buffering agents as well as additives such as saline or glucose may be added to make the solutions isotonic. The drug also may be solubilized in alcohol/propylene glycol or polyethylene glycol for drip intravenous administration. Alternatively, the active ingredients may be in powder form for reconstituting with a suitable vehicle prior to administration.

The term "unit dosage form" as used in the specification and claims refer to physically discrete units, each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the pharmaceutical carrier. Examples of such unit dosage forms are tablets, capsules, pills, powder packets, wafers, measured units in ampoules or in multidose containers and the like. A unit dosage of the present invention may contain from 100 to 1000 milligrams of one of the compounds.

HIV Protease Inhibitors - I

The present invention is also concerned with a compound which inhibits the protease encoded by human immunodeficiency virus (HIV). The compound, or pharmaceutically acceptable salt thereof, is of value in the prevention of infection by HIV, the treatment of infection by HIV and the treatment of the resulting acquired immune deficiency syndrome (AIDS). The present invention also relates to pharmaceutical compositions containing the compounds, and to a method of use of the present compounds with or without other agents for the treatment of AIDS & viral infection by HIV.

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A retrovirus designated human immunodeficiency virus (HIV) is the etiological agent of the complex disease that includes progressive destruction of the immune system (acquired immune deficiency syndrome; AIDS) and degeneration of the central and peripheral nervous system. This virus was previously known as LAV, HTLV-III, or ARV. A common feature of retrovirus replication is the extensive post-translational processing of precursor polyproteins by a virally encoded protease to generate mature viral proteins required for virus assembly and function. Interruption of this processing appears to prevent the production of normally infectious virus. For example, Crawford, S. *et al.*, J. Virol., 53, 899, 1985, demonstrated that genetic deletion mutations of the protease in murine leukemia virus which prevent processing of precursor structural proteins result in non-infectious viral particles. Unprocessed structural proteins also have been observed in clones of non-infectious HIV strains isolated from human patients. These results suggest that inhibition of the HIV protease represents a viable method for the treatment of AIDS and the prevention or treatment of infection by HIV.

Nucleotide sequencing of HIV shows the presence of a pol gene in one open reading frame [L. Ratner, *et al.*, Nature, 313, 277(1985)]. Amino acid sequence homology provides evidence that the pol sequence encodes reverse transcriptase, an endonuclease and an HIV protease [H. Tol, *et al.*, EMBO J. 4, 1267 (1985); M. D. Power, *et al.*, Science, 231, 1567 (1986); L. H. Pearl, *et al.*, Nature 329, 351 (1987)].

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Applicants demonstrate that the compound of this invention is an inhibitor of HIV protease. The compound of this invention provides a prodrug for the inhibition of HIV protease.

5 A biotransformed compound as herein defined is disclosed. This compound is useful in the inhibition of HIV protease, the prevention of infection by HIV, the treatment of infection by HIV and in the treatment of AIDS and/or ARC, either as
10 a compound, pharmaceutically acceptable salt (when appropriate), pharmaceutical composition ingredient, whether or not as a prodrug or as a combination with other antivirals, anti-infectives, immunomodulators, antibiotics or vaccines. Methods of treating AIDS,
15 methods of preventing infection by HIV, and methods of treating infection by HIV are also disclosed.

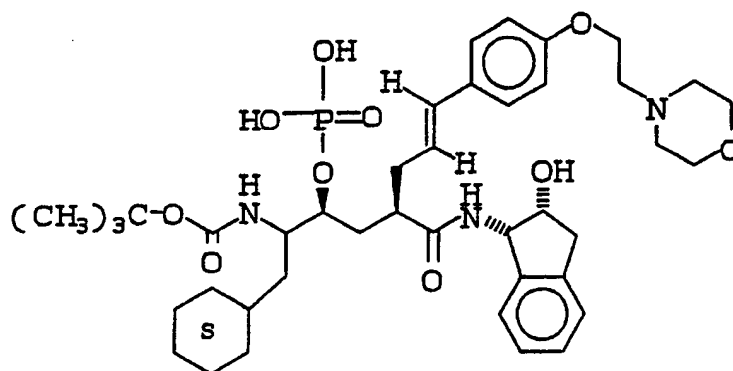
This invention is also concerned with the use of a compound given below, or pharmaceutically acceptable salts thereof, in the inhibition of HIV
20 protease, the prevention or treatment of infection by HIV and in the treatment of the resulting acquired immune deficiency syndrome (AIDS). The biotransformed compound is the product of the incubation of Rhizopus
arrhizus (ATCC 11145) in the presence of L-702,083,
25 an HIV protease inhibitor. It is defined as follows:

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or pharmaceutically acceptable salts, hydrates or
15 esters thereof.

The pharmaceutically-acceptable salts of
the compound of the present invention (in the form
of water- or oil-soluble or dispersible products)
20 include the conventional non-toxic salts or the
quaternary ammonium salts of this compound, which
are formed, e.g., from inorganic or organic acids or
bases. Examples of such acid addition salts include
acetate, adipate, alginate, aspartate, benzoate,
25 benzenesulfonate, bisulfate, butyrate, citrate,
camphorate, camphorsulfonate, cyclopentanepropionate,
digluconate, dodecylsulfate, ethanesulfonate,
fumarate, glucoheptanoate, glycerophosphate,
hemisulfate, heptanoate, hexanoate, hydrochloride,
30 hydrobromide, hydroiodide, 2-hydroxyethanesulfonate,

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lactate, maleate, methanesulfonate, 2-naphthalene-sulfonate, nicotinate, oxalate, pamoate, pectinate, persulfate, 3-phenyl-propionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, 5 tosylate, and undecanoate. Base salts include ammonium salts, alkali metal salts such as sodium and potassium salts, alkaline earth metal salts such as calcium and magnesium salts, salts with organic bases such as dicyclohexylamine salts, N-methyl-D-glucamine, 10 and salts with amino acids such as arginine, lysine, and so forth. Also, the basic nitrogen-containing groups may be quaternized with such agents as lower alkyl halides, such as methyl, ethyl, propyl, and butyl chloride, bromides and iodides; dialkyl 15 sulfates like dimethyl, diethyl, dibutyl; and diamyl sulfates, long chain halides such as decyl, lauryl, myristyl and stearyl chlorides, bromides and iodides, aralkyl halides like benzyl and phenethyl bromides and others. Hydrates or esters are also encompassed 20 by the present invention. Such hydrates or esters are those which would readily occur to the skilled artisan, and include, for example, C₁₋₄alkyl esters.

The compound of the present invention is useful in the inhibition of HIV protease, the 25 prevention or treatment of infection by the human immunodeficiency virus (HIV) and the treatment of consequent pathological conditions such as AIDS. Treating AIDS or preventing or treating infection by HIV is defined as including, but not limited to, 30 treating a wide range of states of HIV infection: AIDS, ARC (AIDS related complex), both symptomatic

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and asymptomatic, and actual or potential exposure to HIV. For example, the compound of this invention is useful in treating infection by HIV after suspected past exposure to HIV by e.g., blood transfusion, accidental needle stick, or exposure to patient blood during surgery.

For these purposes, the compound of the present invention may be administered orally, parenterally (including subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques), by inhalation spray, or rectally, in dosage unit formulations containing conventional non-toxic pharmaceutically-acceptable carriers, adjuvants and vehicles.

Thus, in accordance with the present invention there is further provided a method of treating and a pharmaceutical composition for treating HIV infection and AIDS. The treatment involves administering to a patient in need of such treatment a pharmaceutical composition comprising a pharmaceutical carrier and a therapeutically-effective amount of the compound of the present invention.

These pharmaceutical compositions may be in the form of orally-administrable suspensions or tablets; nasal sprays; sterile injectable preparations, for example, as sterile injectable aqueous or oleagenous suspensions or suppositories.

When administered orally as a suspension, these compositions are prepared according to techniques well-known in the art of pharmaceutical

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formulation and may contain microcrystalline cellulose for imparting bulk, alginic acid or sodium alginate as a suspending agent, methylcellulose as a viscosity enhancer, and sweeteners/flavoring agents known in the art. As immediate release tablets, these compositions may contain microcrystalline cellulose, dicalcium phosphate, starch, magnesium stearate and lactose and/or other excipients, binders, extenders, disintegrants, diluents and lubricants known in the art.

When administered by nasal aerosol or inhalation, these compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents known in the art.

The injectable solutions or suspensions may be formulated according to known art, using suitable non-toxic, parenterally-acceptable diluents or solvents, such as mannitol, 1,3-butanediol, water, Ringer's solution or isotonic sodium chloride solution, or suitable dispersing or wetting and suspending agents, such as sterile, bland, fixed oils, including synthetic mono- or diglycerides, and fatty acids, including oleic acid.

When rectally administered in the form of suppositories, these compositions may be prepared by mixing the drug with a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride

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esters or polyethylene glycols, which are solid at ordinary temperatures, but liquidify and/or dissolve in the rectal cavity to release the drug.

5 Dosage levels of the order of 0.02 to 5.0
or 10.0 grams-per-day are useful in the treatment or
prevention of the above-indicated conditions, with
oral doses two-to-five times higher. For example,
infection by HIV is effectively treated by the
administration of from 10 to 50 milligrams of the
10 compound per kilogram of body weight from one to
three times per day. It will be understood, however,
that the specific dose level and frequency of dosage
for any particular patient may be varied and will
depend upon a variety of factors including the acti-
15 vity of the specific compound employed, the metabolic
stability and length of action of that compound, the
age of the patient, body weight, general health, sex,
diet, mode and time of administration, rate of excre-
tion, drug combination, the severity of the particular
20 condition, and the host undergoing therapy.

The present invention is also directed to
combinations of the HIV protease inhibitor compound
with one or more agents useful in the treatment of
AIDS. For example, the compound of this invention
25 may be effectively administered, whether at periods
of pre-exposure and/or post-exposure, in combination
with effective amounts of other AIDS antivirals,
immunomodulators, anti-infectives, or vaccines.

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HIV Protease Inhibitor - II

The present invention is also further concerned with another compound which inhibits the protease encoded by human immunodeficiency virus (HIV). The compound, or pharmaceutically acceptable salt thereof, is of value in the prevention of infection by HIV, the treatment of infection by HIV and the treatment of the resulting acquired immune deficiency syndrome (AIDS). The present invention also relates to pharmaceutical compositions containing the compound, and to a method of use of the present compound with or without other agents for the treatment of AIDS & viral infection by HIV.

A retrovirus designated human immunodeficiency virus (HIV) is the etiological agent of the complex disease that includes progressive destruction of the immune system (acquired immune deficiency syndrome; AIDS) and degeneration of the central and peripheral nervous system. This virus was previously known as LAV, HTLV-III, or ARV. A common feature of retrovirus replication is the extensive post-translational processing of precursor polyproteins by a virally encoded protease to generate mature viral proteins required for virus assembly and function. Interruption of this processing appears to prevent the production of normally infectious virus. For example, Crawford, S. *et al.*, J. Virol., 53, 899, 1985, demonstrated that genetic deletion mutations of the protease in murine leukemia virus which prevent processing of precursor structural proteins result in non-infectious viral particles. Unprocessed structural proteins also have been observed in clones of non-infectious HIV strains isolated from human

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patients. These results suggest that inhibition of the HIV protease represents a viable method for the treatment of AIDS and the prevention or treatment of infection by HIV.

5 Nucleotide sequencing of HIV shows the presence of a pol gene in one open reading frame [Ratner, L. et al., Nature, 313, 277(1985)]. Amino acid sequence homology provides evidence that the pol sequence encodes reverse transcriptase, an
10 endonuclease and an HIV protease [Toh, H. et al., EMBO J. 4, 1267 (1985); Power, M.D. et al., Science, 231, 1567 (1986); Pearl, L.H. et al., Nature 329, 351 (1987)].

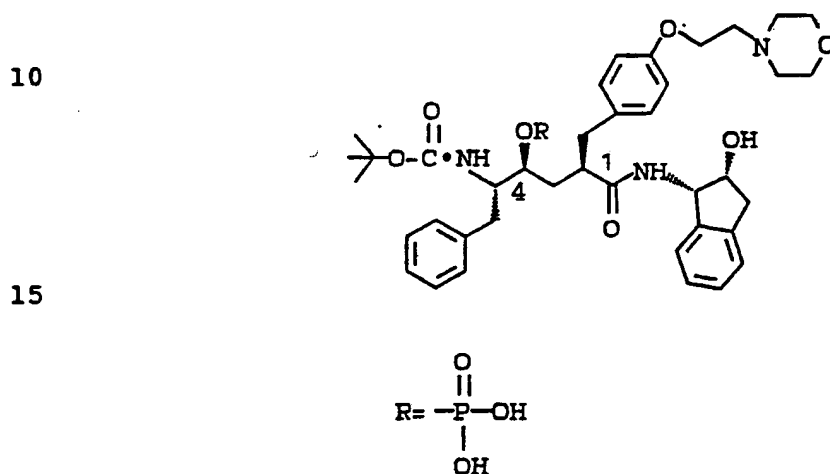
15 Applicants demonstrate that the compound of this invention is an inhibitor of HIV protease. The compound of this invention provides a prodrug for the inhibition of HIV protease.

20 A biotransformed compound as herein defined is disclosed. This compound is useful in the inhibition of HIV protease, the prevention of infection by HIV, the treatment of infection by HIV and in the treatment of AIDS and/or ARC, either as a compound, pharmaceutically acceptable salt (when appropriate), pharmaceutical composition ingredient, whether or
25 not as a prodrug or as a combination with other antivirals, anti-infectives, immunomodulators, antibiotics or vaccines. Methods of treating AIDS, methods of preventing infection by HIV, and methods of treating infection by HIV are also disclosed.

30 This invention is concerned with the use of a compound given below, or pharmaceutically acceptable salts thereof, in the inhibition of HIV protease, the prevention or treatment of infection by HIV and

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in the treatment of the resulting acquired immune deficiency syndrome (AIDS). The biotransformed compound is the product of the incubation of Rhizopus arrhizus (ATCC 11145) in the presence of L-689,502, a
 5 HIV protease inhibitor. It is defined as follows:



or pharmaceutically acceptable salts, hydrates or esters thereof.

The pharmaceutically-acceptable salts of
 25 the compound of the present invention (in the form of water- or oil-soluble or dispersible products) include the conventional non-toxic salts or the quaternary ammonium salts of this compound, which are formed, e.g., from inorganic or organic acids or
 30 bases. Examples of such acid addition salts include acetate, adipate, alginate, aspartate, benzoate,

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benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalene-sulfonate, nicotinate, oxalate, pamoate, pectinate, persulfate, 3-phenyl-propionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, and undecanoate. Base salts include ammonium salts, alkali metal salts such as sodium and potassium salts, alkaline earth metal salts such as calcium and magnesium salts, salts with organic bases such as dicyclohexylamine salts, N-methyl-D-glucamine, and salts with amino acids such as arginine, lysine, and so forth. Also, the basic nitrogen-containing groups may be quaternized with such agents as lower alkyl halides, such as methyl, ethyl, propyl, and butyl chloride, bromides and iodides; dialkyl sulfates like dimethyl, diethyl, dibutyl; and diamyl sulfates, long chain halides such as decyl, lauryl, myristyl and stearyl chlorides, bromides and iodides, aralkyl halides like benzyl and phenethyl bromides and others. Hydrates or esters are also encompassed by the present invention. Such hydrates or esters are those which would readily occur to the skilled artisan, and include, for example, C₁₋₄alkyl esters.

The compound of the present inventions is useful in the inhibition of HIV protease, the prevention or treatment of infection by the human immunodeficiency virus (HIV) and the treatment of

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consequent pathological conditions such as AIDS. Treating AIDS or preventing or treating infection by HIV is defined as including, but not limited to, treating a wide range of states of HIV infection:

5 AIDS, ARC (AIDS related complex), both symptomatic and asymptomatic, and actual or potential exposure to HIV. For example, the compound of this invention is useful in treating infection by HIV after suspected

10 past exposure to HIV by e.g., blood transfusion, accidental needle stick, or exposure to patient blood during surgery.

For these purposes, the compound of the present invention may be administered orally, parenterally (including subcutaneous injections,

15 intravenous, intramuscular, intrasternal injection or infusion techniques), by inhalation spray, or rectally, in dosage unit formulations containing conventional non-toxic pharmaceutically-acceptable carriers, adjuvants and vehicles.

20 Thus, in accordance with the present invention there is further provided a method of treating and a pharmaceutical composition for treating HIV infection and AIDS. The treatment involves administering to a patient in need of such treatment a phar-

25 maceutical composition comprising a pharmaceutical carrier and a therapeutically-effective amount of the compound of the present invention.

These pharmaceutical compositions may be in the form of orally-administrable suspensions

30 or tablets; nasal sprays; sterile injectable preparations, for example, as sterile injectable aqueous or oleagenous suspensions or suppositories.

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When administered orally as a suspension, these compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may contain microcrystalline cellulose for imparting bulk, alginic acid or sodium alginate as a suspending agent, methylcellulose as a viscosity enhancer, and sweeteners/flavoring agents known in the art. As immediate release tablets, these compositions may contain microcrystalline cellulose, dicalcium phosphate, starch, magnesium stearate and lactose and/or other excipients, binders, extenders, disintegrants, diluents and lubricants known in the art.

When administered by nasal aerosol or inhalation, these compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents known in the art.

The injectable solutions or suspensions may be formulated according to known art, using suitable non-toxic, parenterally-acceptable diluents or solvents, such as mannitol, 1,3-butanediol, water, Ringer's solution or isotonic sodium chloride solution, or suitable dispersing or wetting and suspending agents, such as sterile, bland, fixed oils, including synthetic mono- or diglycerides, and fatty acids, including oleic acid.

When rectally administered in the form of suppositories, these compositions may be prepared by

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mixing the drug with a suitable non-irritating
excipient, such as cocoa butter, synthetic glyceride
esters or polyethylene glycols, which are solid at
ordinary temperatures, but liquidify and/or dissolve
5 in the rectal cavity to release the drug.

Dosage levels of the order of 0.02 to 5.0
or 10.0 grams-per-day are useful in the treatment or
prevention of the above-indicated conditions, with
oral doses two-to-five times higher. For example,
10 infection by HIV is effectively treated by the
administration of from 10 to 50 milligrams of the
compound per kilogram of body weight from one to three
times per day. It will be understood, however, that
the specific dose level and frequency of dosage for
15 any particular patient may be varied and will depend
upon a variety of factors including the activity of
the specific compound employed, the metabolic stability
and length of action of that compound, the age
of the patient, body weight, general health, sex,
20 diet, mode and time of administration, rate of
excretion, drug combination, the severity of the
particular condition, and the host undergoing therapy.

The present invention is also directed to
combinations of the HIV protease inhibitor compound
25 with one or more agents useful in the treatment of
AIDS. For example, the compound of this invention
may be effectively administered, whether at periods
of pre-exposure and/or post-exposure, in combination
with effective amounts of other AIDS antivirals,
30 immunomodulators, anti-infectives, or vaccines.

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Simvastatin Analogs

Also included by the process of this invention are two phosphorylated derivatives of simvastatin, whose chemical name is 6(R)-[2-(8'
5 (S)-2'',2''-dimethylbutanoyloxy-2'(S),6'(R)-dimethyl-
1',2',6',7',8',8'a (R)-hexahydronaphthyl-1'(S)-
ethyl]-4(R)-hydroxy-3,4,5,6-tetrahydro-2H-pyran-
2-one. See EPO Publication No. 0033 358 and The
10 Merck Index, Eleventh Edition, for a description
of the compound, its synthesis and utility as an
anti-hypercholesterolemic agent in interfering
with cholesterol biosynthesis.

Contacting simvastatin with the Rhizopus
microorganism under the conditions described
15 herein results in two phosphorylated derivatives:

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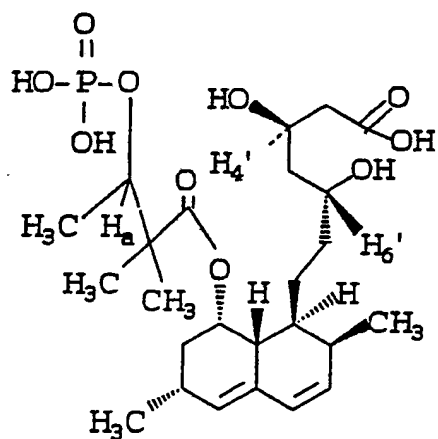
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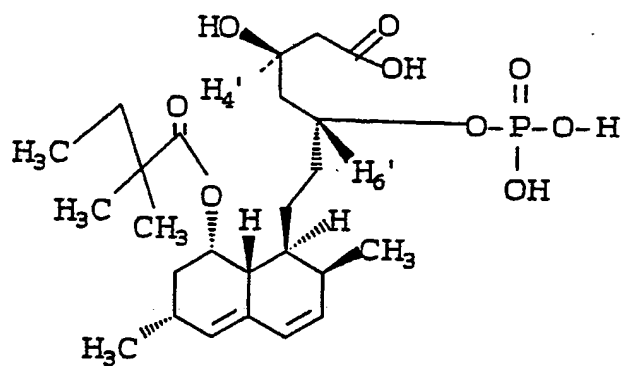


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L-706, 526

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L-706, 527

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The above two compounds of this invention are useful as antihypercholesterolemic agents for the treatment of atherosclerosis, hyperlipemia and like diseases in humans. They may be administered orally or parenterally in the form of a capsule, a tablet, an injectable preparation or the like. It is usually desirable to use the oral route. Doses may be varied, depending on the age, severity, body weight and other conditions of human patients but daily dosage for adults is within a range of from about 2 mg to 2000 mg (preferably 10 to 100 mg) given in three or four divided doses. Higher doses may be favorably applied as required.

The compounds of this invention also have useful anti-fungal activities. For example, they may be used to control strains of *Penicillium* sp., *Aspergillus niger*, *Cladosporium* sp., *Cochliobolus miyabeanus* and *Helminthosporium cynodnotis*. For those utilities they are admixed with suitable formulating agents, powders, emulsifying agents or solvents such as aqueous ethanol and sprayed or dusted on the plants to be protected.

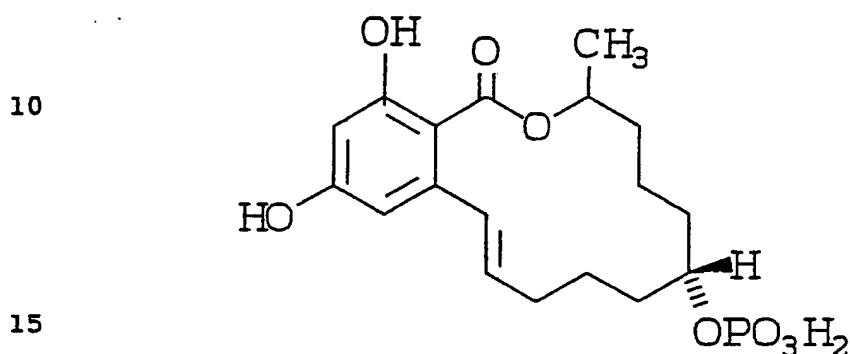
The pharmaceutically acceptable salts of this invention include those formed from cations such as sodium, potassium, aluminum, calcium, lithium, magnesium, zinc and tetramethylammonium as well as those salts formed from amines such as ammonia, ethylenediamine, N-methylglucamine, lysine, arginine, ornithine, choline, N,N'-dibenzylethylenediamine, chloroprocaine, diethanolamine, procaine, N-benzylphenethylamine, 1-p-chlorobenzyl-2-pyrrolidine-1'-yl-methylbenzimidazole, diethylamine, piperazine, and tris(hydroxymethyl)aminomethane.

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Zearalenone

Also included by the process of this invention is the biotransformation product: alpha zearalenol 6'-phosphate:

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α -zearalenol-6' -phosphate

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The present invention also relates to the above new compound and an object of the present invention is to provide compounds which exhibit estrogenic activity or aid in increasing the rate of growth in meat-producing animals, e.g. cattle, lamb and swine.

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The compound can be administered to animals by any suitable method including oral and parenteral administrations. For example, the compound can be blended with ordinary feed containing nutritional values in an amount sufficient to produce the desired rate of growth and can thus be fed directly to the

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animals, or the compound can be suspended in a suitable injection suspension medium such as peanut oil and injected parenterally. The amount of compound fed to an animal, of course, varies depending upon
5 the animal, desired rate of growth and the like.

When the new products are to administered in feeds, an animal feed composition may be prepared containing the usual nutritionally-balanced quantities of carbohydrates, proteins, vitamins and minerals,
10 together with the compounds of the present invention. Some of these usual dietary elements are grains, such as ground grain and grain by-products; animals protein substances, such as those found in fish meal and meat scraps; vegetable proteins like
15 soybean oil meal or peanut oil meal; vitaminaceous materials, e.g. vitamin A and D complex members; and bone meal and limestone to provide minerals. A type of conventional feed material for use with cattle includes alfalfa hay and ground corn cobs together
20 with supplementary vitaminaceous substances is desired.

The starting material racemic (\pm)-zearalenone, a potent anabolic agent useful in the raising of meat-producing animals, originally was
25 prepared by fermenting the microorganism, Gibberella zeae (Gordon), on a suitable nutrient medium according to the techniques described in U.S. Patent No. 3,169,019. More recently there has been
described the total chemical synthesis of racemic (\pm)-
30 zearalenone and the 2,4-dimethyl ether derivative

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thereof (Taub et al., Chemical Communications, 1967, p. 225). The process described in U.S. Patent No. 3,551,455 and in U.S. Patent No. 3,239,545 affords new and more direct routes to the total synthesis of
5 (+)-zearalenones.

Example IA

A spore sand culture containing Gibberella
10 zeae (Gordon) NRRL-2830 was aseptically placed in a sterile tube containing 15 milliliters of Czapek's-Dox solution and a small amount of agar. This medium was then incubated for about 168 hours at approximately 25°C. At the end of the incubation period,
15 the medium was washed with 5 milliliters of sterile deionized water and transferred to a sterile tube containing 45 milliliters of Czapek's-Dox solution. The contents of the tube were then incubated for about 96 hours at about 25°C after which the material
20 was available for use in inoculation of a fermentation medium.

The following example illustrates the fermentation of the organism Gibberella zeae (Gordon) NRRL-2830 to produce zearalenone.
25

Example IIA

To a 2 liter flask were added 300 grams of finely divided corn. The flask and its contents were then sterilized and after sterilization 150
30 milliliters of sterile deionized water were added.

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To the mixture in the flask were then added 45 milliliters of the inoculum prepared by the process of Example IA and the material was thoroughly mixed. The mixed material was then incubated for about 20 days at 25°C in a dark room in a water-saturated atmosphere.

The following example illustrates the recovery of the zearalenone from the fermentation medium.

10

Example IIIA

A 300 gram portion of fermented material produced by the method of Example IIA was placed in 500 milliliters of deionized water and slurried. The slurry was then heated for about 15 minutes at 75°C, 300 grams of filter aid were then added and the material was filtered. The solid filtered material containing the anabolic substance was then air dried, and 333 grams of the dried cake were then extracted with 500 milliliters of ethanol. This procedure was repeated three more times. The ethanol extract was evaporated to dryness under vacuum to give 6.84 grams of solid material. This solid material was then dissolved in 20 milliliters of chloroform and extracted with 30 milliliters of an aqueous solution containing 5% by weight of sodium carbonate having an adjusted pH of about 11.2. The extraction process was repeated seven more times. The pH of the sodium carbonate extract was then adjusted to 6.2 with hydrochloric acid, to yield an anabolic substance-containing precipitate. The precipitate and the aqueous sodium

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carbonate extract were then each in turn extracted with 75 milliliters of ethyl ether. This procedure was repeated three more times to yield a light yellow ethereal solution, which was then evaporated to yield
5 116 milligrams of solid anabolic substance. This material was then subjected to multiple transfer countercurrent distribution using 100 tubes and a solvent system consisting of two parts chloroform and two parts carbontetrachloride as the lower phase and
10 four parts methanol and one part water as the upper phase, all parts by volume. The solid material obtained from the multiple transfer countercurrent distribution was zearalenone.

The following examples are given for the
15 purpose of illustrating the present invention and should not be construed as being limitations on the scope or spirit of the instant invention.

EXAMPLE 1

20 C-32 Phosphorylated FK-520 Microorganism and Culture Conditions

Spores of Rhizopus oryzae MF4974 (ATCC No. 11145) cultivated on an oatmeal agar flask were inoculated into 50 ml Soy-Glucose medium in a 250 ml
25 Erlenmer flask and shaken at 27°C on a rotary shaker at 220 rpm for 24 hours. The second stage flask (50 ml in a 250 ml Erlenmeyer flask) were inoculated with 2.5 ml of seed culture and incubated on a rotary shaker (220 rpm) at 27°C for 24 hours. Following
30 incubation, each flask was harvested by

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centrifugation, washed once with sterile water, and resuspended in equal volume of 100 mM pH 7.0 PO_4 buffer containing 3% glycerol. FK-520 was added to achieve a final concentration of 0.2 mg/ml. The
5 flasks were then incubated on a rotary shaker (220 rpm) at 27°C for 48 hours. Following incubation, the whole broth was extracted as described in the Isolation/Purification Section below.

10	<u>Media</u>	<u>Soy Glucose Medium</u>	<u>g/l</u>
		Dextrose	20.0
		Soy Meal	5.0
		Fido yeast extract	5.0
		NaCl	5.0
15		K_2HPO_4	5.0
		Adjust pH to 7.0	

ISOLATION AND PURIFICATION

The whole broth (400 ml) was adjusted to
20 pH 3.5 and extracted three times with methylene chloride (3 x 400 ml). Methylene chloride extracts were combined and evaporated to dryness under reduced pressure at 30°C. The resulting oil was dissolved in methanol and subjected to HPLC purification. HPLC
25 was carried out on Whatman Partisil 10 ODS-3, 9.4 mm x 25 cm at 50°C and monitored at 205 nm. The column was developed at 3 ml/minutes with a linear gradient from 35% acetonitrile in 0.1% phosphoric acid to 80% in 0.1% phosphoric acid in 60 minutes. The compound
30 was collected during repeated injections of the above described extract. The fractions at retention time, 32 minutes, were pooled, adjusted to pH 3 and

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evaporated to remove acetonitrile. The compound was further purified using C18 Sep Pak (Water Associates) and methanol-water elution solvent to yield 4.5 mg pf product.

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Characterization

C-32 Phosphorylated FK-520 (FK-900520) was characterized by FAB mass spectrometry and FK-520 via NMR spectrometry yielding the proton NMR spectrum of Figure 1, which confirms the assigned molecular structure in Figure 2.

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EXAMPLE 2

T-Cell Proliferation Assay

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1. Sample Preparation

Purified C-32 phosphorylated FK-520, as prepared by HPLC above, was dissolved in absolute ethanol at 10 mg/ml.

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2. Assay

Spleens from C57Bl/6 mice were taken under sterile conditions and gently dissociated in ice-cold RPMI 1640 culture medium (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (GIBCO). Cells were pelleted by centrifugation at 1500 rpm for 8 minutes. Contaminating red cells were removed by treating the pellet with ammonium chloride lysing buffer (GIBCO) for 2 minutes at 4°C. Cold medium was added and cells were again centrifuged at 1500 rpm for 8 minutes. T lymphocytes were then isolated by separation of the cell suspension on nylon wool columns as follows: Nylon wool columns

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were prepared by packing approximately 4 grams of washed and dried nylon wool into 20 ml plastic syringes. The columns were sterilized by autoclaving at 250°F for 30 minutes. Nylon wool columns
5 were wetted with warm (37°C) culture medium and rinsed with the same medium. Washed spleen cells resuspended in warm medium were slowly applied to the nylon wool. The columns were then incubated in an upright position at 37°C for 1 hour. Non-adherent
10 T lymphocytes were eluted from the columns with warm culture medium and the cell suspensions were spun as above.

Purified T lymphocytes were resuspended at 2.5×10^5 cells/ml in complete culture medium composed
15 of RPMI 1640 medium with 10% heat-inactivated fetal calf serum, 100 mM glutamine, 1 mM sodium pyruvate, 2×10^{-5} M 2-mercaptoethanol and 50 µg/ml gentamycin. Ionomycin was added at 250 ng/ml and PMA at 10 ng/ml. The cell suspension was immediately distributed into
20 96 well flat-bottom microculture plates (Costar) at 200 µl/well. The control, being the medium without test drug, and various below-indicated dilutions of the above sample of purified C-32 phosphorylated
25 FK-520 to be tested were then added in triplicate wells at 20 µl/well. FK-520 was used as a standard. The culture plates were then incubated at 37°C in a humidified atmosphere of 5% CO₂-95% air for 44 hours. The proliferation of T lymphocytes was assessed by measurement of tritiated thymidine
30 incorporation. After 44 hours of culturing, the cells were pulse-labelled with 2 µCi/well of tritiated thymidine (NEN, Cambridge,

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MA). After another 4 hours of incubation, cultures were harvested on glass fiber filters using a multiple sample harvester. Radioactivity of filter discs corresponding to individual wells was measured by standard liquid scintillation counting methods (Betacounter). Mean counts per minute of replicate wells were calculated and the results expressed as percent inhibition of tritiated thymidine uptake (proliferation) as follows:

$$\% \text{ Inhibition} = 100 - \left[\frac{\text{Mean cpm sample tested}}{\text{Mean cpm control medium}} \times 100 \right]$$

The results of % inhibition at various concentrations of C-32 phosphorylated FK-520 (C-32-P FK-520) are presented in the following table:

TABLE
Inhibition of T-Cell Proliferation
by C-32-P FK-520

	<u>C-32-P-FK-520 (ng/ml)</u>	<u>% Inhibition</u>
	100	97
25	50	95
	25	89.6
	12.5	71.3
	6.2	28.7
30	3.1	0.0

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- Notes:
1. Mouse T cell cultures were pulsed with ^3H -thymidine for 4 hours prior to harvesting at 48 hours.
 2. Standard FK-520 (4 ng/ml) gave 97% inhibition.
 3. The mean IC_{50} for C-32-P FK-520 was determined to be: 15.0 ± 2.1 ng/ml (17.2 ± 2.4 nM) in 3 independent experiments.
 4. Inhibition of T-Cell proliferation was reversed by the addition of 100 units/ml of recombinant human IL-2 at the initiation of culture.

EXAMPLE 3

C-32 Phosphorylated FK-506 Fermentation

Spores of Rhizopus oryzae MF 4974, cultivated on oatmeal agar, were inoculated into 50 ml Soy-Glucose medium in a 250 ml Erlenmeyer flask and shaken at 25°C on a rotary shaker at 220 rpm for 24 hours. The second stage flasks (50ml in a 250 ml Erlenmeyer flask) were inoculated with 2.5 ml of seed culture and incubated on a rotary shaker (220 rpm) at 27°C for 24 hours.

Following incubation, each flask was harvested by centrifugation, washed once with sterile water, and resuspended in equal volume of 100 mM P04 buffer containing 3% glycerol, FK506 was added to achieve a final concentration of 0.2 mg/ml. The

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charged flasks were incubated on a rotary shaker (220 rpm) at 27°C for 24 hours. Following incubation, the whole broth was worked up as described below.

5	<u>Media</u>	<u>Soy Glucose Medium</u>	<u>g/L</u>
		Dextrose	20.0
		Soy meal	5.0
		Fido yeast extract	5.0
		NaCl	5.0
10		K ₂ HPO ₄	5.0

Adjust pH to 7.0

ISOLATION AND PURIFICATION

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The whole broth (200 ml) was maintained at pH 6.8 and centrifuged. The mycelial cake was washed with water, then discarded. The clear filtrate and washings were pooled and passed thru a Spe-ed
20 octadecyl cartridge (14% carbon load, Applied Separations) under vacuum. The column was washed with 100 ml of water. Column effluent and wash did not contain microbial transformation product when tested with HPLC. The cartridge was eluted with 200
25 mL methanol. Methanol was evaporated to dryness under reduced pressure at 30°C. The resulting oil was dissolved in methanol and subjected to HPLC purification.

HPLC was carried out on Whatman Magnum 20
30 Partisil 1- ODS-3 Column (C₁₈, 22.1 mm ID x 25 cm) at 50°C and monitored at 205 nm. The column was developed at 7 mL/min with linear gradient from

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35% acetonitrile in 0.1% phosphoric acid to 80%
acetonitrile in 0.1% phosphoric acid in 70 minutes.

The compound was collected during repeated
injections of the above described extract. Fractions
5 of retention time 50 minutes were pooled, adjusted to
pH 3.0 and evaporated to remove acetonitrile. The
compound was desalted using a C₁₈ Sep Pak (Water
Associate) to yield 20 mg of product.

10 CHARACTERIZATION

The compound was characterized by MS and
confirmed by NMR as the C-32 phosphate ester
derivative of FK 506.

15

EXAMPLE 4

C-32 Phosphorylated C-31-desmethyl FK-520

The fermentation procedure of Example 3 was
carried out substantially identical except that C-31
20 desmethyl FK-520 (available as for example, by the
procedure in EPO Publication 0 349 061, published
January 3, 1990) was used in place of FK-506.

The isolation/purification procedures were
virtually identical to those described in Example 3.

25 The C-32 phosphorylated C-31 desmethyl
FK-520 was characterized by mass spectrometry and
proton nuclear magnetic resonance in which the
obtained spectra was consistent with the assigned
structure.

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EXAMPLE 5

Carrying out the above IL-2 assay described
above in Example 2 for C-32 phosphorylated FK-506
(P-FK-506) and C-32 phosphorylated C-31 desmethyl
FK-520 (P-31-desMe FK-520) yielded the following
results:

Inhibition of T cell proliferation by P-FK-506		
	Concentration of P-FK-506 (mg/ml)	% of inhibition
	50	98.5
	25	97.7
	12.5	93.3
	6.2	80.3
	3.1	42.9

IC₅₀ = 3.4 ng/mL (3.9nM)

Inhibition of T cell proliferation by P-31-desMe FK-520		
	Concentration of P-31-desMe FK-520 (mg/ml)	% of inhibition
	250	95.2
	125	90.3
	62.5	79.5
	31.2	53.8
	15.6	22.2

IC₅₀ = 31.3 ng/mL (36.7 nM).

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EXAMPLE 6Phosphorylated Rapamycin MacrolideMicroorganism and Culture Conditions

Spores of Rhizopus oryzae MF4974 (ATCC
 5 No. 11145) cultivated on an oatmeal agar flask were
 inoculated into 50 ml Soy-Glucose medium in a 250 ml
 Erlenmer flask and shaken at 27°C on a rotary shaker
 at 220 rpm for 24 hours. The second stage flask (500
 10 ml in a 1000 ml Erlenmeyer flask) were inoculated
 with 25 ml of seed culture and incubated on a rotary
 shaker (220 rpm) at 27°C for 24 hours. Following
 incubation, each flask was harvested by centrifuga-
 tion, washed once with sterile water, and resuspended
 15 in equal volume of 100 mM pH 7.0 phosphate buffer con-
 taining 3% glycerol. Rapamycin macrolide was added
 to achieve a final concentration of 0.2 mg/ml. The
 flasks were then incubated on a rotary shaker (220
 rpm) at 27°C for 24 hours. Following incubation,
 the whole broth was extracted as described in the
 20 Isolation/Purification Section below.

<u>Media</u>	<u>Soy Glucose Medium</u>	<u>g/l</u>
	Dextrose	20.0
	Soy Meal	5.0
25	Fido yeast extract	5.0
	NaCl	
5.0		
	K ₂ HPO ₄	5.0
	Adjust pH to 7.0	

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ISOLATION AND PURIFICATION

The whole broth (500 ml) was maintained at pH 6.8 and centrifuged. The mycelial cake was washed with water, then discarded. The clear filtrate and washings were pooled and passed through a Speed octadecyl cartridge (14% carbon load, Applied Separations) under vacuum. The column was washed with 100 ml of water. Column effluent and wash did not contain microbial transformation product when tested with HPLC. The cartridge was eluted with 200 ml methanol. Methanol was evaporated to dryness under reduced pressure at 30°C. The resulting oil was dissolved in methanol and subjected to HPLC purification. HPLC was carried out on Whatman Magnum 9 Partisil 10 ODS-3, 9.8 mm i.d. x 25 cm at 25°C and monitored at 225 nm. The column was developed at 3 ml/minutes with a linear gradient from 35% to 80% acetonitrile in 0.1% phosphoric acid in 30 minutes. The compound was collected during repeated injections of the above described extract. The fractions of retention time 17.3 minutes, were pooled, adjusted to pH 3 and evaporated to remove acetonitrile. The compound was further purified using C18 Sep Pak (Water Associates) and methanol-water elution solvent to yield 14 mg pf product.

Characterization

The C-43 phosphorylated macrolide was characterized by FAB mass spectrometry and NMR spectrometry as the methyl phosphate ester derivative yielding the proton NMR spectrum of Figure 3, which confirms the assigned molecular structure in Figure 4.

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Methylation to produce the phosphate ester was necessary to minimize the considerable line broadening which characterized the proton NMR spectrum of the free acid. The key features were the downfield displaced H-42 and H-43 signals (which are H-31 and H-32 in FK-506 nomenclature) at 3.87 ppm and 4.12 ppm, respectively, and the additional fine structure of H-43 resulting from coupling with the phosphorus. The downfield shift of H-43 is a normal consequence of replacing the active hydrogen with an electron withdrawing substituent. H-42 is also displaced downfield by proximity to the new substituent. The obtained spectrum of the phosphorylated macrolides as the phosphate methyl ester with assignments of H-42, H-43, and the methyl ester peaks is shown in Figure 3.

EXAMPLE 7

T-Cell Proliferation Assay

1. Sample Preparation

Purified phosphorylated macrolide, as prepared by HPLC above, was dissolved in absolute ethanol at 1 mg/ml and serially diluted in culture medium prior to addition to the cultures.

2. Assay

The assay was carried out as described in J. Immunol. Vol. 144, pp.251 (1990) by F. Dumont, et al.

Spleens from C57Bl/6 mice were taken under sterile conditions and gently dissociated in ice-cold RPMI 1640 culture medium (GIBCO, Grand Island, N.Y.)

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supplemented with 10% heat-inactivated fetal calf serum (GIBCO). Cells were pelleted by centrifugation at 1500 rpm for 8 minutes. Contaminating red cells were removed by treating the pellet with ammonium chloride lysing buffer (GIBCO) for 2 minutes at 4°C. Cold medium was added and cells were again centrifuged at 1500 rpm for 8 minutes. T lymphocytes were then isolated by separation of the cell suspension on nylon wool columns as follows: Nylon wool columns were prepared by packing approximately 4 grams of washed and dried nylon wool into 20 ml plastic syringes. The columns were sterilized by autoclaving at 250°F for 30 minutes. Nylon wool columns were wetted with warm (37°C) culture medium and rinsed with the same medium. Washed spleen cells resuspended in warm medium were slowly applied to the nylon wool. The columns were then incubated in an upright position at 37°C for 1 hour. Non-adherent T lymphocytes were eluted from the columns with warm culture medium and the cell suspensions were spun as above.

Purified T lymphocytes were resuspended at 2.5×10^5 cells/ml in complete culture medium composed of RPMI 1640 medium with 10% heat-inactivated fetal calf serum, 100 mM glutamine, 1 mM sodium pyruvate, 2×10^{-5} M 2-mercaptoethanol and 50 µg/ml gentamycin. Human recombinant interleukin-2 (50 µg/ml) and PMA at 10 ng/ml. The cell suspension was immediately distributed into 96 well flat-bottom microculture plates (Costar) at 200 µl/well. The control, being the medium without test drug, and various below-indicated dilutions of the above sample of purified phosphorylated macrolide to be tested were then

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added in triplicate wells at 20 μ l/well. Rapamycin (U.S. Patent 3,929,992) was used as a standard. The culture plates were then incubated at 37°C in a humidified atmosphere of 5% CO₂-95% air for 44 hours.

5 The proliferation of T lymphocytes was assessed by measurement of tritiated thymidine incorporation. After 44 hours of culturing, the cells were pulse-labelled with 2 μ Ci/well of tritiated thymidine (NEN, Cambridge, MA). After another 4 hours of incubation,

10 cultures were harvested on glass fiber filters using a multiple sample harvester. Radioactivity of filter discs corresponding to individual wells was measured by standard liquid scintillation counting methods (Betacounter). Mean counts per minute of replicate

15 wells were calculated and the results expressed as percent inhibition of tritiated thymidine uptake (proliferation) as follows:

$$20 \quad \% \text{ Inhibition} = 100 - \left[\frac{\text{Mean cpm sample tested}}{\text{Mean cpm control medium}} \times 100 \right]$$

Proliferation was assessed at 48 hours of culture by tritiated thymidine uptake.

25 The results, expressed as IC₅₀ values of three independent experiments, are presented in the following table:

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TABLE
Inhibition of T-Cell Proliferation
IC₅₀ (ng/ml)

5		Phosphorylated	
	<u>Expt. #</u>	<u>Rapamycin</u>	<u>Rapamycin</u>
10	1	7.7	0.6
	2	7.3	0.4
	3	7.9	0.3

-
- Notes:
1. Mouse T cell cultures were pulsed with ³H-thymidine for 4 hours prior to harvesting at 48 hours.
 2. The mean IC₅₀ for the C-43 phosphorylated rapamycin was determined to be: 7.6 ± 0.2 ng/ml and 0.4 ± 0.1 ng/ml for rapamycin in 3 independent experiments.

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EXAMPLE 8SYNTHESIS

The following preparation and synthesis
5 follows, in general, U.S. Patent 4,661,473; Evans,
B.E. et al., J. Org. Chem., 50, 4615, (1985) and
Evans, B.E. et al., "A Stereocontrolled Synthesis of
Hydroxyethylene Dipeptide Isosteres," Proc. Am. Pept.
Symp., 9, 743-6(1985), and Luly, J.R. et al., J. Org.
10 Chem, 52, 1487 (1987).

N(2(R)-Hydroxy-1(S)indanyl)-5(S)-((1,1-dimethylethoxy-
carbonyl)amino)-4(S)-hydroxy-6-phenyl-2(R)-3-(4-(2-
15 (morpholino)ethoxy)phenyl)prop-2-en-1-yl)hexanamide:

Step A: Preparation of 4-tert-butyldimethylsilyloxy-
phenylprop-2-en-1-yl bromide:

To a 1 L round bottomed flask with a stirring
bar and an argon inlet was added 26.25 g (160 mmol)
20 of p-hydroxycinnamic acid, 50.62 g (335 mmol) of tert-
butyldimethylsilyl chloride, 32.68 g (480 mmol) of
imidazole, and 250 mL of dry DMF. This mixture was
stirred at room temperature for 24 hours. The DMF
was removed in vacuo and the residue was partitioned
25 between EtOAc and 10% aqueous citric acid. The layers
were separated and the organic phase was washed with
water (4x) and brine. Drying (MgSO₄), filtration and
removal of the solvent in vacuo gave 62.3 g of the
bissilylether-ester. This ester was placed in an
30 oven dried 2 L round bottomed, 3 necked flask with
a mechanical stirrer, argon inlet, and addition
funnel. Ether (460 mL) was added and the solution

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was cooled in an ice bath to 0°C. To this solution was added a solution of Dibal-H 397 mL of a 1.0 M solution in hexanes), dropwise over 1 hour. The mixture was stirred for 1 hour and the reaction was then quenched by careful addition of 1 L of saturated aqueous sodium potassium tartrate solution. This viscous mixture was stirred for 20 hours at room temperature. The mixture was filtered through a celite pad, the filtrate layers were separated and the organic phase was extracted with 2 portions of EtOAc. The combined organic phases were washed with brine, dried (MgSO₄), filtered and concentrated in vacuo to give the crude alcohol. This material was chromatographed on 500 g of silica gel using 20% EtOAc in hexanes as eluant. There was obtained 34.9 g of 4-tert-butyldimethylsilyloxycinnamyl alcohol as a viscous oil. This material crystallized on cooling to -15°C. A portion of this alcohol (18.5 g, 69.96 mmol) was placed in a 300 mL round bottomed flask with a stirring bar and 125 mL of dry ether. This solution was cooled to 0°C and 20.83 g (76.95 mmol) of PBr₃ was added dropwise with a syringe over 5 minutes. This solution was maintained at 0°C for 20 minutes, diluted with hexanes (1L) and washed with aqueous NaHCO₃. This solution was washed with brine, dried (MgSO₄), filtered through a pad of silica gel, and concentrated in vacuo to give 17.53 g of 4-tert-butyldimethylsilyloxypenylprop-2-en-1-yl bromide as a colorless oil.

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Step B: Preparation of 5(S)-(1,1-dimethylethoxycarbonyl)amino-4(S)-(1',1'-dimethylethyl-1,1-dimethylsilyloxy)-6-phenyl-2(R)-(4-(1',1'-dimethylsilyloxy)phenylprop-2-en-yl)hexanoic acid:

5

To a 500 mL, 3-necked, oven dried round bottomed flask with an argon inlet, stirring bar, low temperature thermometer, and a jacketed addition funnel was added 75 mL of dry THF and 15.39 mL (109.78 mmol) of diisopropylamine. This solution was cooled to -20°C and n-butyllithium (42.84 mL, 107.11 mmol of a 2.5M solution in hexanes) was added slowly. The resulting solution was cooled to -78°C and a solution of (5S,1'S)-5-((1,1-dimethylethoxycarbonyl)amino)-2-phenylethyl) dihydrofuran-2-(3H)-one (16.36 g, 53.55 mmol) in 75 mL of dry THF was added at such a rate that the temperature of the solution did not rise above -70°C (ca. 40 minutes required for this addition). The resulting solution was aged at -78°C for 1 hour and the dropping funnel was charged with a solution of 4-tert-butyldimethylsilyloxyphenylprop-2-en-1-yl bromide (17.53 g, 53.55 mmol) in 75 mL of dry THF. The bromide solution was cooled to -78°C and was then added to the enolate solution dropwise over 45 minutes, keeping the temperature below -70°C. When the addition was complete the solution was aged at -78°C for 1 hour, warmed to -50°C and quenched with a solution of NaHSO₄ (33 g) in 250 mL of water. The mixture was diluted with EtOAc and the layers were separated. The organic phase was washed with NaHCO₃ solution and brine. Drying (MgSO₄), filtration, and removal of the solvent in vacuo

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provided the crude alkylation product as an oil. This material was chromatographed on 1000 g of silica gel using 20% EtOAc in hexanes as eluant. There was obtained 23.6 g of pure (5S,3R,1'S)3-

5 (4-(1',1'-dimethylethyl-1,1-dimethylsilyloxy)phenyl-prop-2-en-1-yl)-5-(1-((1,1-dimethylethoxycarbonyl)-amino)-2-phenylethyl)dihydrofuran-2-(3H)-one as a colorless foam. This material was dissolved in 325 mL of DME and a solution of LiOH (6.99 g, 291.97

10 mmol) in H₂O (325 mL) was added. This solution was stirred at room temperature for 24 hours. The DME was removed in vacuo and the aqueous residue was acidified with 10% aqueous citric acid. This milky suspension was extracted with several portions of

15 EtOAc and the combined extracts were washed with water and brine, dried (MgSO₄), filtered, and concentrated in vacuo. To the crude α -hydroxy acid was added imidazole (56.79 g, 834 mmol), tert-butyl-dimethylsilyl chloride (62.87 g, 417 mmol) and dry

20 DMF (325 mL). This mixture was stirred at room temperature for 24 hours. The mixture was then treated with methanol (325 mL) for 4 hours at room temperature. The solvents were removed in vacuo at 60°C, 20 torr. The residue was dissolved in 1L of

25 EtOAc and washed with 10% aqueous citric acid, water and brine. Drying (MgSO₄), filtration and removal of the solvent gave 28.53 g of 5(S)-(1,1-dimethylethoxycarbonylamino)-4(S)-(1',1'-dimethylethyl-1,1-dimethyl-

30 silyloxy)-6-phenyl-2(R)-(4-(1',1'-dimethylethyl-1,1-dimethylsilyloxy)phenylprop-2-en-1-yl)hexanoic acid as a colorless foam.

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Step C: Preparation of N-(2(R)-hydroxy-1(S)-indanyl)-5(S)-(1,1-dimethylethoxycarbonylamino)-4(S)-(1',1'-dimethylethyl-1,1-dimethylsilyloxy)-6-phenyl-2(R)-((4-(hydroxyphenyl)prop-2-en-1-yl)hexanamide:

To a 2L round bottomed flask with a stirring bar and an argon inlet was added 5(S)-(1,1-dimethylethylethoxycarbonylamino)-4(S)-(1',1'-dimethylethyl-1,1-dimethylsilyloxy)-6-phenyl-2(R)-((4(1',1'-dimethylethyl-1,1-dimethylsilyloxy)phenylprop-2-en-1-yl)hexanoic acid (28.53 g, 41.71 mmol) from Step B, 2(R)-hydroxy-1(S)-aminoindane (6.85 g, 45.88 mmol), 3-(N,N-dimethylaminopropyl)ethylcarbodiimide hydrochloride (8.79 g, 45.8 mmol), 1-hydroxybenztriazole hydrate (6.20 g, 45.88 mmol), and dry DMF (300 mL). When all of the solids had dissolved, triethylamine (12.79 mL, 91.76 mmol) was added and the mixture was stirred at room temperature for 18 hours. The mixture was partitioned between EtOAc (1500 mL) and 10% aqueous citric acid (1500 mL). The layers were separated and the organic phase was washed with water (3 x 1000 mL), and brine. Drying (MgSO₄), filtration and removal of the solvent in vacuo gave 32 g of a yellow foam. This material was dissolved in 500 mL of methanol and LiOH (4.99 g, 208.55 mmol) was added. This solution was stirred at room temperature for 1 hour. The solution was acidified with aqueous citric acid and the methanol was removed in vacuo. The resulting aqueous residue was extracted with EtOAc (1L). The EtOAc extract was dried (MgSO₄), filtered, and concentrated in vacuo. This material was chromatographed on 1 Kg of silica gel using 8L of 40%

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EtOAc in hexanes as eluant. There was obtained 20.07 g of N-(2(R)-hydroxy-1(S)-indanyl)-5(S)-(1,1-dimethylethoxycarbonyl)amino-4(S)-(1',1'-dimethylethyl-1,1-dimethylsilyloxy)-6-phenyl-2(R)-((4-hydroxyphenyl)prop-2-en-1-yl)hexanamide as a colorless foam.

Step D: Preparation of N-(2(R)-hydroxy-1(S)-indanyl)-5(S)-(1,1-dimethylethoxycarbonylamino)-4(S)-hydroxy-6-phenyl-2(R)-((4-(2-(4-morpholino)-ethoxy)phenyl)prop-2-en-1-yl)hexanamide:

To a 1L round bottomed flask with a stirring bar and an argon inlet was added N-(2(R)-hydroxy-1(S)-indanyl)-5(S)-(1,1-dimethylethoxycarbonyl)-4(S)-(1',1'-dimethylethyl-1,1-dimethylsilyloxy)-6-phenyl-2(R)-((4-(1',1'-dimethylethyl-1,1-dimethylsilyloxy)phenyl)prop-2-en-1-yl)hexanamide (19.49 g, 28.1 mmol) 1,4-dioxane (400 mL), 4-(2-chloroethyl)morpholine (12.61 g, 84.3 mmol), and powdered cesium carbonate (27.5 g, 84.3 mmol). This mixture was heated at 80°C with vigorous stirring for 3 hours. The cooled reaction mixture was filtered through a celite pad and the 1,4-dioxane was removed in vacuo. To this residue was added a solution of tetrabutylammonium fluoride in THF (280 mL of a 1M solution, 280 mmol) and a stirring bar. This solution was stirred at room temperature under argon for 28 hours. The reaction mixture was poured into 3L of H₂O, with stirring. The white solid was collected by filtration and dried in vacuo overnight. The crude product was chromatographed on silica gel using 5% MeOH in chloroform as eluant. The chromatographed material was recrystallized from boiling EtOAc-hexanes to give

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13 g of analytically pure N-(2(R)-hydroxy-1(S)-indanyl)-5(S)-(1,1-dimethylethoxycarbonylamino)-4(S)-hydroxy-6-phenyl-2(R)-(4-(2-(4-morpholino)ethoxy)-phenylprop-2-en-1-yl)hexanamide as a white solid,
5 mp:178-179°C.

EXAMPLE 9

Preparation of N-(2R)-hydroxy-1(S)-indanyl)-5(S)-
10 (1,1-dimethylethoxyloxycarbonylamino)-4(S)-hydroxy-
2(R)-(4-(2-(4-morpholino)ethoxy)phenyl)prop-2-en-1-
yl-6-cyclohexylhexanamide, parent compound L-702,083

15 Step A: Preparation of (5S,1'S)-5-(1'-((1,1-di-
methylethoxycarbonyl)amino)-2'-cyclo-
hexylethyl)-4,5-dihydrofuran-2-(3H)-one:

A solution of the (5S,1'S)-5-(1'-((1,1-di-
methylethoxycarbonyl)amino)-2'-phenylethyl)-4,5-
dihydrofuran-2-(3H)-one was dissolved in ethyl
20 acetate and rhodium on alumina was added. This
mixture was shaken under a hydrogen atmosphere (50
psi) at 50°C overnight. Filtration and evaporation
of the solvent afforded the title compound as a
viscous oil, which solidified as a hard glass.

25

Step B: Steps B, C and D of Example 1 are repeated
except that (5S, 1'S)-5-((1,1-dimethylethoxy-
carbonyl)amino)-2-phenylethyl)dihydrofuran-2-
(3H)-one in Step B is substituted with its
30 cyclohexyl analog, (5S, 1'S)-5-((1-dimethyl-
ethoxycarbonyl)amino)-2-cyclohexylethyl)-
4,5-dihydrofuran-2-(3H)-one. The title
compound, L-702,083, is obtained.

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EXAMPLE 10BIOTRANSFORMATION OF L-702,083

5 Spores of Rhizopus arrhizus MF 4974,
cultivated on oatmeal agar, were inoculated into 50
ml of seed medium [containing(in grams per liter)
dextrose 20.0, soy meal 5.0, Fidco yeast extract 5.0,
10 NaCl 5.0, K₂HPO₄ 5.0, pH 7.0 before autoclaving] in a
250 ml 3-baffle Erlenmeyer flask. The flask was
incubated for 24 hours on a rotary shaker (220 rpm)
at 27°C. The 2.5 ml of the seed cultures was used to
inoculate 50 ml of the same medium in a 250 ml
Erlenmeyer flask and incubated on a rotary shaker
15 (220 rpm) at 27°C for 24 hours. Following
incubation, each flask was harvested by
centrifugation, washed once with sterile saline
solution, and resuspended in equal volume of 100 mM
phosphate buffer (pH 7.0) containing 3% glycerol.
20 L-702,083 in DMSO (20 mg/ml) was then added to
achieve a final concentration of 0.1 mg/ml, and
cultivation was continued at 27°C on a rotary shaker
at 220 rpm. After 12 hours incubation the whole
broth was extracted as described in Example 16.

25

EXAMPLE 11ISOLATION AND PURIFICATION

30 Two shake flasks were combined to yield 100
ml of broth. The broth was centrifuged. The
mycelial cake was washed with water and discarded.
The clear filtrate and the wash were combined and

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passed thru a Spe-ed octadecyl 14% cartridge (Applied Separations) under vacuum. The column was washed with 100 ml of water and then eluted with 200 ml methanol. Methanol extract was concentrated in vacuo to a slightly oily residue. The residue was taken up in methanol and subjected to preparative HPLC chromatography on a Whatman Magnum 20 Partisil 10 ODS-3, 22.1 mm x 25 cm at room temperature at a flow rate of 7 ml/minute. The effluent stream was monitored by U.V. absorption at 215 nm. Elution was achieved with a linear gradient from 30% acetonitrile in 0.1% phosphoric acid to 80% acetonitrile in 0.1% phosphoric acid in 80 minutes. The fractions at retention time of 35 minutes were combined, adjusted to pH 4.0 and evaporated to remove acetonitrile. The compound was then desalted using C-18 Sep Pak (Waters Associate) to afford 3 mg of product L-706,579.

EXAMPLE 12

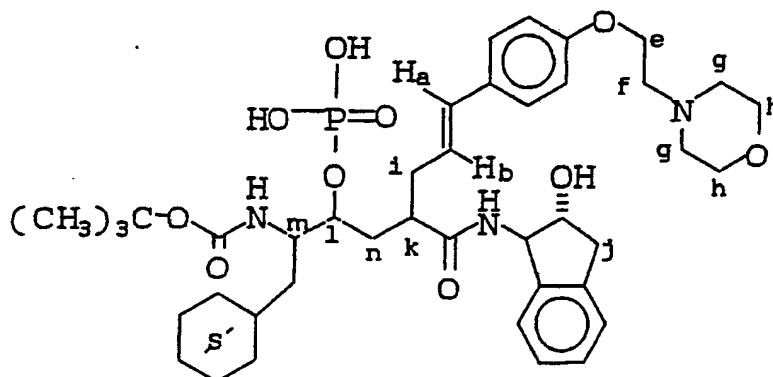
NMR CHARACTERIZATION

The product of Example 11, L-706,579, was characterized by NMR as the phosphate ester derivative at C-4 of L-702,083. The key observation was the 0.6 ppm downfield displacement of H₁ in the Formula :

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5

10



Compared to its chemical shift in the parent L-702,083, the neighboring methine (m) is slightly displaced downfield and is barely discernible as a shoulder at the base of the morpholine CH₂O at 3.74 ppm. Also perturbed is the nearby H_k which now appears underneath the lower field H_j signal at 3.10 ppm. Its presence is revealed only by the additional area of the H_j signal, compared with the H_j doublet in L-702,083.

EXAMPLE 13

ORGANIC SYNTHESIS OF L-706,579, PHOSPHATE ESTER
OF L-702,083

A. Acetylation of Indan

N-[(2R)-Acetoxy-1(S)-indanyl]-5(S)-(1,1-dimethyl-ethoxycarbonylamino)-4(S)-hydroxy-2(R)-[(4-(2-(4-

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morpholino)ethoxy)phenyl)prop-2-en-1-yl]-6-cyclo-
hexanamide, L-702,083, is prepared by the protocol of
Examples 1 and 2, except that 2(R)-hydroxy-1(S)-
aminoindan is substituted with 2(R)-acetoxy-1(S)-
5 aminoindan in Example 1, Step C.

B. L-706,579

The product of step A is reacted with
10 monophenyl phosphorodichloridate according to the
principles and practice of Chambers, R.W. and H.G.
Khorana, J. Am. Chem. Soc. 80, 3749 (1958).
Subsequent treatment with excess ammonia, followed by
removal of the acetyl groups under basic conditions,
15 yields L-706,579.

C. Alternative Synthetic Routes

Another phosphorylating agent is dibenzyl
phosphorochloridate. An extensive discussion of
20 phosphorylation and phosphorylating agents can be
found in Y. Mizuno, Studies in Org. Chem, 24, 171-175
(1986) and references cited therein.

25

30

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EXAMPLE 14

Assay for Inhibition of Recombinant
HIV Protease

5

Inhibition studies were performed on the reaction of the HIV protease expressed in Escherichia coli with a tritiated peptide substrate [³H]-acetyl-Val-Ser-Gln-Asn-(beta-naphthyl-Ala)-Pro-Ile-Val-Gln-
10 Gly-Arg-Arg-NH₂ (MW 1800). The two arginine residues at the carboxyl terminus give this peptide an overall positive charge at acidic pH and enable it to bind to the H⁺ form of DOWEX AG-50W-X8 resin and similar resins. The HIV protease cleaves between the
15 B-naphthyl-Ala and proline residues to yield a product (³H-acetyl-val-ser-asn-(beta-naphthyl-ala) that is either neutral or slightly negatively charged and does not bind to the cation exchange resin. It is therefore possible to conveniently separate the
20 labelled product from the substrate.

Aliquots of 25 µl containing 6.0-8.0 nM HIV protease in assay buffer (100 mM sodium acetate, pH 5.5 and 0.1% BSA) are placed in assay tubes. The reaction is initiated by addition of 25 µl aliquots
25 of 4.2 µM tritiated peptide substrate in 100mM sodium acetate, pH 5.5. After incubation for 60 min at 37°C, the reaction is stopped with 100 µl of 5% H₃PO₄, then analysed by application of column chromatography.

30

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Results are as follows:

	<u>Conc. L-706.579(μM)</u>	<u>%Inhibition</u>
	100	100
5	10	89
	1	20
	0.1	3
	0.01	0
	0.001	
10		

EXAMPLE 15SYNTHESIS OF L-689.502

The preparation and synthesis follows, in
15 general, U.S. Patent 4,661,473; Evans, B.E. et al,
J. Org. Chem., 50, 4615, (1985) and Evans, B.E. et
al., "A Stereocontrolled Synthesis of Hydroxyethylene
Dipeptide Isosteres," Proc. Am. Pept. Symp., 2,
743-6(1985), and Luly, J.R. et al, J. Org. Chem, 52,
20 1487 (1987), all herein incorporated by reference.
All temperatures are in degrees centigrade, unless
indicated otherwise.

Preparation of N-(cis-2(R)-hydroxy-1(S)-indanyl)-
25 5(S)-(1,1-dimethylethoxycarbonylamino)-4(S)-hydroxy-
6-phenyl-2(R)-[(4-(2-(4-morpholinyl)ethoxy)phenyl)
methyl]-hexanamide. L-689.502

Step A: Preparation of N-3(S)-[(1,1-Dimethylethoxy-
30 carbonyl)amino]-2(RS)-hydroxy-4-phenyl-1-tri-
methylsilyl butane:

To a stirred suspension of magnesium turnings

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(9.79 g, 403 mmol) in dry diethyl ether (200 mL) under nitrogen was added chloromethyltrimethylsilane (50 mL, 358 mmol). The reaction was initiated by gentle warming and then was cooled in an ice bath to maintain gentle reflux. After exotherm was complete the reaction was stirred at room temperature for 1 hour then cooled to -78°C in a dry ice/acetone bath. To the solution of the Grignard was added dropwise with stirring a solution of N-2(S)-[(1,1-dimethylethoxycarbonyl)amino]-3-phenyl propionaldehyde (19.3 g, 77.4 mmol) in dry diethyl ether (250 mL) dropwise such that the temperature of the reaction remained below -55°C . The resultant gray suspension was allowed to warm to room temperature where it was stirred for 30 minutes then was quenched by pouring into a mixture of ice (500 g) and 10% citric acid (500 mL). The organic phase was collected and the aqueous phase was extracted with diethyl ether (3 X 300 mL). The combined organics were washed with 10% citric acid (1 X 300 mL) and brine (1 X 200 mL), dried over anhydrous magnesium sulfate, filtered, and concentrated to give crude N-3(S)-[(1,1-dimethylethoxycarbonyl)amino]-2(RS)-hydroxy-4-phenyl-1-trimethylsilyl butane (26.6 g, quantitative crude yield) as a yellow oil. An analytical sample was obtained by low pressure chromatography (silica gel, 230-400 mesh; diethyl ether: hexanes, 30%:70%) followed by recrystallization from heptane. mp = $91-95^{\circ}\text{C}$; elemental analysis. Calcd. for $\text{C}_{18}\text{H}_{31}\text{NO}_3\text{Si}$ (337.53):
C = 64.05, H = 9.26, N = 4.15;
Found: C = 64.05, H = 9.13, N = 4.22; $[\alpha]_{\text{D}}^{20} = -40.0^{\circ}$.

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Step B: Preparation of 3(S)-Amino-4-phenyl-1-butene.

To a stirred solution of the product of Step A (22.8 g, 67.5 mmol) in dry methylene chloride (400 mL) cooled in an ice bath and under nitrogen was added in a fine stream boron trifluoride etherate (43 mL, 345 mmol). The solution was allowed to warm to room temperature where it was stirred for 4 days. Reaction was cooled in an ice bath and quenched by the dropwise addition of 10% sodium hydroxide (400 mL). The organic phase was collected and the aqueous phase was extracted with methylene chloride (2 X 250 mL). The combined organics were washed with brine (1 X 200 mL), dried over anhydrous magnesium sulfate, filtered, and concentrated to give crude 3(S)-amino-4-phenyl-1-butene (14.2 g) as a yellow oil.

Step C: Preparation of N-3(S)-[(1,1-Dimethylethoxycarbonyl)amino]-4-phenyl-1-butene:

A solution of the product of Step B (14.2 g) and di-~~tert~~-butyl dicarbonate (31.0 g, 142 mmol) in dry methylene chloride (200 mL) was stirred at room temperature for 18 hours, washed with 10% citric acid (3 X 100 mL), water (1 X 100 mL), sat'd. sodium bicarbonate (3 X 125 mL), and brine (1 X 250 mL), dried over anhydrous magnesium sulfate, filtered and concentrated to yield crude N-3(S)-1[(1,1-dimethylethoxycarbonyl)amino]-4-phenylbutene (34.6 g) as a yellow oil. Crude product was purified by low pressure chromatography (silica gel, 230-400 mesh, 10 X 20 cm column; diethylether: hexanes, 20%: 80%) to yield N-3(S)-[(1,1-dimethylethoxycarbonyl)amino]-4-phenyl-1-butene (16.3 g, 97.6% yield) as a white

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solid. An analytical sample was obtained by recrystallization from heptane. mp = 67.5-68.5°C; elemental analysis, Calcd. for $C_{15}H_{21}NO_2$ (247.34):

C = 72.84, H = 8.56, N = 5.66.

5 Found: C = 72.78, H = 8.76, N = 5.64.

Step D: Preparation of 1(R)-[1'(S)-(1,1-Dimethylethoxycarbonyl)amino-2-phenylethyl]oxirane:

To a solution of the product of Step C
10 (9.4 g, 38 mmol) in dry methylene chloride (100 mL) cooled in an ice bath and under nitrogen was added 3-chloroperoxybenzoic acid (technical grade, 80-85%; 41 g, 200 mmol). The mixture was stirred at 0°C for 18 hours and 25°C for 23 hours, then diluted with
15 diethyl ether (300 mL), and poured in ice cold aqueous 10% sodium sulfite (1 L). The organic layer was collected and the aqueous layer was extracted with diethyl ether (3 X 100 mL). The combined organics were washed with 10% sodium sulfite (3 X 100 mL),
20 satd. sodium bicarbonate (3 X 100 mL), and brine (1 X 100 mL), dried over anhydrous sodium sulfate, filtered and concentrated to give a white solid. Crude product was purified by low pressure chromatography (silica gel 230 - 400 mesh, 8 X 15 cm
25 column; ethyl acetate: hexanes, 25%:75%) to yield 1(R)-[1'(S)-(1,1-dimethylethoxycarbonyl)amino-2-phenylethyl]oxirane (7.0 g, 70% yield) as a clear oil which crystallized upon standing. An analytical sample was obtained by recrystallization from heptane.
30 mp = 51.5-52°C; elemental analysis, Calcd. for $C_{15}H_{21}NO_2$ (263.34):

C = 68.42, H = 8.04, N = 5.32.

Found: C = 68.22, H = 8.26, N = 5.29; $[\alpha]_D^{20} = 1.34^\circ$.

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Step E: Preparation of (5S,1'S)-3-carboethoxy-5-(1-((1',1'-dimethylethoxycarbonyl)amino)-2-phenylethyl)-dihydrofuran-2-(3H)-one.

The product from Step D, 9.93 g, was
5 dissolved in 100 mL of absolute ethanol and added to a solution of 2.6 g of sodium and 20.1 mL of diethyl malonate in 170 mL of absolute ethanol. After stirring overnite, the reaction was acidified to pH 4 with 10% citric acid and extracted with 2 X 500 mL of
10 ether. The combined organic extracts were washed 1 X 500 mL H₂O, 1 X 500 mL sat'd NaHCO₃, 1 X 500 mL sat'd brine and dried over MgSO₄. The solvents were removed and the crude product purified by low pressure chromatography on silica gel eluting with 50% ether/hexanes
15 (or EtOAc/hexanes). The yield of semi-solid product was 10.6 g. The later fractions contained 2.5 g of the undesired 5 R isomer as a white solid.

Step F: Preparation of (5S,1'S)-3-carboethoxy-3-(4-benzyloxyphenylmethyl)-5-[1-(1,1-dimethylethoxycarbonyl)amino)-2-phenylethyl]dihydrofuran-2-(3H)-one

To a stirred solution of (5S,1'S)-3-carboethoxy-5-[1-((1',1'-dimethylethoxycarbonyl)amino)-7-phenylethyl)-dihydrofuran-2-(3H)-one (product of Step
25 E), 2 g (5.3 mmol) in 25 mL of absolute ethanol was added a solution of 0.13 g of sodium in 2.2 mL of absolute ethanol followed by 1.30 g (5.5 mmol) of 4-benzyloxybenzyl chloride. The solution was heated
30 to 50°C under nitrogen for 1 hour, then cooled in an ice bath and acidified with 20 mL of 10% citric acid and diluted with 200 mL of water. The mixture was

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extracted with 3 X 100 mL of ether and the combined ether extracts washed with 50 mL of water, 200 mL of sat'd NaHCO₃ and dried over MgSO₄. Removal of solvents under reduced pressure and purification by
5 low pressure chromatography on silica gel, eluting with 40% ether in hexanes gave 1.56 g (51% yield) of a clear colorless glass essentially homogeneous by TLC (50% ether/hexanes).

10 Step G: Preparation of (3R,5S,1'S)-3-(4-benzyloxy-phenylmethyl)-5-(1((1,1-dimethylethoxy-carbonyl)amino)-2-phenylethyl)-dihydrofuran-2-(3H)-one.

The product of Step F, 13.6 g, was dissolved
15 in 250 mL of 1,2-dimethoxyethane, and to it was added 117 mL of 1 M lithium hydroxide at room temperature. After stirring for 12 hours, the solvents were removed under reduced pressure, the residue suspended in 200 mL of 10% citric acid and extracted 3 X 500 mL of
20 diethyl ether. The combined ether extracts were washed with 500 mL of brine, dried (MgSO₄) and the concentrated to dryness. The residue was dissolved in 250 mL of toluene, heated to reflux for 12 hours, then concentrated to dryness under reduced pressure.
25 Purification by medium pressure chromatography over silica gel eluting with 15% ethyl acetate/hexanes gave 3.2 g of the 3R-lactone as a clear foam. Further elution with the same solvents gave 6.15 g
30 of the 3S-lactone as a white solid.

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Step H: Preparation of N'-(1,1-dimethylethoxy-carbonyl)-5(S)-amino-4(S)-(1',1'-dimethylethyl-1,1-dimethylsilyloxy)-6-phenyl-2(R)-(4-benzyloxyphenylmethyl-hexanoic acid.

5 The product of Step G, 0.6 g, was dissolved in 30 mL of a 2:1 mixture of ethylene glycol dimethyl ether/water, and to it was added 5 mL of 1 M lithium hydroxide at room temperature. After stirring for 1 hour, the mixture was partitioned between 200 mL
10 chloroform and 20 mL 10% citric acid. The layers were separated and the aqueous phase extracted with 3 X 20 mL chloroform. The combined organic layers were dried (Na_2SO_4) and the solvent removed to yield 0.56 g of the crude hydroxy acid. This residue was
15 dissolved in 5 mL of dry DMF and 0.845 g tert-butyl dimethylsilyl chloride and 0.725 g of imidazole were added. After stirring for 18 hours, the reaction was poured into 50 mL of water and extracted with 3 X 20 mL of ethyl acetate. The combined organic extracts
20 were washed with 3 X 20 mL of 10% citric acid, 1 X 20 mL of water, 3 X 10 mL of saturated aqueous solution of Na_2CO_3 , and 20 mL of brine. After drying (Na_2SO_4), the solvent was removed and the resulting residue dissolved in a mixture of 5 mL of THF, 5 mL of glacial
25 acetic acid, and 2 mL of water. The mixture was stirred for 4 hours, then poured into 50 mL of water and extracted with 3 X 20 mL of ether. The combined ether extracts were washed with 2 X 20 mL of water, brine, dried (Na_2SO_4), and the solvent removed.
30 Purification by medium pressure chromatography over silica gel, eluting with $\text{MeOH}/\text{CHCl}_3$ gave 0.60 g of the product as a white glassy solid.

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Step I: Resolution of 1-Amino-2-hydroxyindan

From the known racemic 1-amino-2-hydroxyindan, the resolution was carried out as described for the 3-amino-1,2-dihydroxyindan in Example 7 below (Steps D and E). The (1S,2R)-1-amino-2-hydroxyindan resulting from saponification of the higher R_f diastereomer was shown to have an a_D of -58° ($c = 1.0$, CHCl_3). The (1R, 2S)-1-amino-2-hydroxyindan resulting from saponification of the lower R_f diastereomer was found to have an a_D of $+62^\circ$ ($c = 1.0$, CHCl_3).

Step J: Preparation of N-(2(R)-hydroxy-1(S)-indanyl)-5(S)-(1,1-dimethylethoxycarbonylamino)-4(S)-hydroxy-6-phenyl-2(R)-(4-benzyloxyphenyl-methyl) hexanamide

The product from Step H, 0.12 g, was dissolved in 2 ml dry DMF and to it was added 40 mg of 1(S)-amino-2(R)-hydroxyindane, (Step I) 25 mg of 1-hydroxybenzotriazole hydrate and 70 mg of dimethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride. Triethylamine was added to the stirred solution until the pH was 8.5 (32 mL). After stirring for concentrated to dryness under reduced pressure, the residue was dissolved in 100 mL of chloroform and worked with 1 X 50 mL of 10% citric acid, 1 X 50 mL H_2O , 1 X 50 mL sat'd NaHCO_3 , dried over MgSO_4 and concentrated to dryness. The residue was dissolved in 1 mL of tetrahydrofuran and added to 2 mL of 1 M tetrabutyl-ammonium fluoride in THF. After stirring overnight at room temperature the reaction mixture was diluted with 10 mL of 10% citric acid and the white preci-

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pitrate collected by filtration. The product was purified by low pressure chromatography on silica gel eluting with 2% methanol/CH₂Cl₂ to give 85 mg of product which was essentially homogeneous by TLC
5 (3% methanol/CH₂Cl₂).

Step K: Preparation of N-(2(R)-hydroxy-1(S)-indanyl)-5(S)-(1,1-dimethylethoxycarbonylamino)-4(S)-hydroxy-6-phenyl-2(R)-(4-hydroxyphenyl-
10 methyl)hexanamide

The product of Step J, 85 mg was dissolved in 10 mL of methanol and 10 mL of THF, and to it was added 0.10 g of 10% palladium on carbon. The mixture was stirred under an atmosphere of hydrogen for 48
15 hours at room temperature, then filtered and concentrated to dryness. The residue was dissolved in 10 mL of hot ethanol and 20 mL water was added. On cooling the white solid precipitate was collected and dried under vacuum over P₂O₅. The yield was 72 mg (98%
20 yield) of pure product: mp 218-219°C (effervesces, sinters at 215) elemental analysis, Calc'd for C₃₃H₄₀N₂O₆: (560.696):
C, 70.69; H, 7.19; N, 5.00;
Found: C, 70.62; H, 7.39; N, 4.79.
25

Step L: Preparation of N-(cis-2(R)-hydroxy-1(S)-indanyl)-5(S)-[1,1-dimethylethoxycarbonylamino)-4(S)-hydroxy-6-phenyl-2(R)-[(4-(2-(4-morpholinyl)ethoxy)phenyl)methyl]-
30 hexanamide

A stirred mixture of Step K product, N-(2(R)-hydroxy-1(S)-indanyl)-5(S)-[1,1-dimethylethoxy-

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carbonyl)amino]-4(S)-hydroxy-6-phenyl-2(R)-(4-hydroxy-phenylmethyl) hexanamide (0.50 g, 0.9 mmol), anhydrous cesium carbonate (1.0 g, 3 mmol) and N-(2-chloroethyl) morpholine, free base (2.35 g, 17 mmole) in 100 mL
5 of anhydrous dioxane was heated to 80°C (internal temperature) for 3 hrs. After cooling to room temperature the mixture was diluted with chloroform (50 mL) filtered, concentrated to dryness under reduced pressure, and the residue triturated with
10 50 ml of anhydrous ether and 10 mL of ethyl acetate. The white solid product was collected and dried under vacuum over P₂O₅. The yield was 0.54 g (89%) of pure product L-689,502: mp 195-7°C. elemental analysis, Calc'd. for C₃₉H₅₁N₃O: (673.856):

15

C, 69.52 H, 7.63; N, 6.23;
Found: C, 69.19 H, 7.45; N, 6.15.

maleate hydrate:
20

mp 112-113°C dec. elemental analysis,
Calc'd. for C₃₉H₅₁N₃O₇.C₄H₄O₄.H₂O: (807.946):
C, 63.92 H, 7.11; N, 5.20;
Found: C, 64.23 H, 6.94; N, 5.10.

25

EXAMPLE 16

A. Fermentation of L-689.502

A frozen vial (2.0 ml) of Rhizopus arrhizus
30 MF4974 was used to inoculate a 250 ml baffled shake flask containing 50 ml of seed medium A. The seed flask was incubated on a rotary shaker (220 rpm) at

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27°C for 24 hours. A 2.5 ml aliquot of the developed seed was used to inoculate a 250 ml non-baffled flask containing 50 ml of transformation medium B; L-689,502 in DMSO was added to the fermentation at 0 hour to
5 achieve a final concentration of 0.05 mg/ml. The shake flask contents were subsequently incubated at 27°C on a rotary shaker for 4 days. The resultant whole broth was extracted as described in Section B.

10	<u>Media:</u>	<u>Seed Medium A</u>	<u>g/l</u>
		Dextrose	1.0
		Dextrin	10.0
		Beef Extract	3.0
		Ardamine pH	5.0
15		NZ Amine Type E	5.0
		MgSO ₄ •7H ₂ O	0.05
		K ₂ HPO ₄	0.3
		Adjust pH to 7.1	
		Add to CaCO ₃ 0.5 g/l	
20		<u>Transformation Medium B</u>	<u>g/l</u>
		Glucose	10
		Hycase SF	2
		Beef Extract	1
25		Corn Steep Liquor	3
		Adjust pH to 7.0	

B. Isolation and Purification

30 The whole broth (400 ml) was extracted three times with 1-butanol (3 x 400 ml). The extracts were combined and concentrated under vacuum to an oily residue. The residue was dissolved in methanol and

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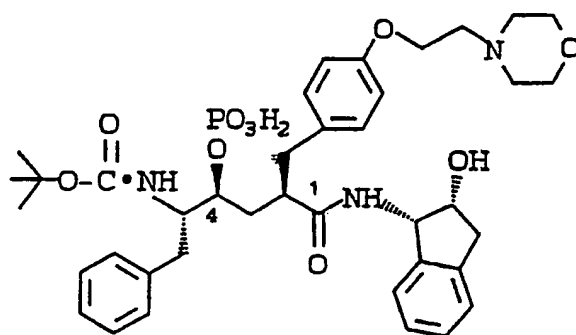
subjected to high performance liquid chromatography (HPLC). HPLC was carried out on Whatman Partisil 10 ODS-3, 9.4 mm x 25 cm at room temperature and monitored at 215 nm. The column was developed at 3 ml/min with a linear gradient from 0.1% aqueous H_3PO_4 - CH_3CN , 80:20, to 0.1% aqueous H_3PO_4 - CH_3CN , 20:80, in 60 minutes. The compound was collected during repeated injections of the above described extract. The fractions at retention time, 19.3 minutes, were pooled, adjusted to pH 6.5 and evaporated to remove acetonitrile. The compound was further purified using a C_{18} Sep-Pak (Waters Associates) and methanol-water elution solvent to yield 11 mg of product.

Anal. Calc'd. for $\text{C}_{39}\text{H}_{50}\text{O}_{10}\text{PN}_3\text{Na}_2$:

C, 58.72 H, 6.27; N, 5.27; P, 3.89.

Found: C, 59.78 H, 6.58; N, 4.97; P, 4.17.

NMR, elemental analysis, and MS showed the structure to be



L-696,432

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Phosphorus was identified and quantified by complexometric and gravimetric methods. Phosphorylation at C₄ was established by NMR from the 0.5 ppm downfield displacement of the C₄H relative to its chemical shift in the parent L-689,502.

EXAMPLE 17

Enzymatic Treatment of L-696,432

The microbial transformation metabolite L-696,432 was determined to be a phosphate ester of L-689,502, a highly potent HIV protease inhibitor. To determine if the phosphate ester bond can be enzymatically cleaved, treatment with alkaline phosphatase was performed.

To 0.5 ml of water containing 0.8 mg of L-696,432 was added 30 microliter of bacterial alkaline phosphatase (0.31 unit/microliter), and the reaction mixture was incubated at 37°C and monitored by HPLC. The reaction was complete after four hours of incubation. The reaction product was purified by HPLC and subjected to FAB MS analysis. [M+H]⁺ ion was observed at m/z 674. The fragmentation ions at m/z 574, 556, 469 and 425 confirmed the product is L-689,502.

The results show that L-696,432 can be enzymatically hydrolyzed back to the parent compound L-689,502.

30

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EXAMPLE 18Assay for Inhibition of Recombinant HIV Protease

5 Inhibition studies of the reaction of HIV
protease expressed in Escherichia coli were carried
out with a tritiated peptide substrate, [³H]-acetyl-
Val-Ser-Gln-Asn-(beta-naphthyl Ala)-Pro-Ile-Val-Gln-
10 Gly-Arg-Arg-NH₂ (MW=1800). The two arginine residues
at the carboxyl terminus give this peptide an overall
positive charge at acidic pH and enable it to bind
to the H⁺ form of DOWEX AG-50W-X8 resin and similar
resins. The HIV protease cleaves between the
15 B-naphthyl-Ala and proline residues to yield a product
[³H]-acetyl-val-ser-asn-(B-naphthyl-ala) that is
either neutral or slightly negatively charged and
does not bind to the cation exchange resin. It is
therefore possible to conveniently separate the
labelled product from the substrate.

20 Aliquots of 25 µl containing 6.0-8.0 nM HIV
protease in assay buffer (100 mM sodium acetate, pH
5.5 and 0.1% BSA) are placed in assay tubes. The
reaction is initiated by addition of 25 µl aliquots
of 4.2 µM tritiated peptide substrate in 100 mM sodium
25 acetate, pH 5.5. After incubation for 60 minutes at
37°C, the reaction is stopped with 100 µl of 5% H₃PO₄,
then analysed by application of column chromatography.

 L-696,432 demonstrated 32% inhibition at a
concentration of 1 µg/ml in substantially purified
30 form.

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EXAMPLE 19

Organic Synthesis of L-696.432.
phosphate ester of L-689.502

5

Organic Synthesis of L-696.432

A. Acetylation of Indan

10 N-(cis-2(R)-Acetoxy-1(S)indanyl)-5(S)-[1,1-dimethylethoxycarbonylamino)-4(S)-hydroxy-6-phenyl-2(R)-[(4-(2-(4-morpholinyl)ethoxy)phenyl)methyl]-hexanamide is prepared by the protocol of Example 1, except that 1(S)-amino-2(R)-acetoxyindane substitutes for 1(S)-amino-2(R)-hydroxyindane of step I.

15

B. L-696.432

The product of step A is reacted with monophenyl phosphorodichloridate according to the principles and practice of Chambers, R.W. and H.G. Khorana, J. Am. Chem. Soc. 80, 3749 (1958). Subsequent treatment with excess ammonia, followed by removal of the acetyl and phenyl groups under basic conditions, yields L-696,432.

20

25 C. Alternative Synthetic Routes

Another phosphorylating agent is dibenzyl phosphorochloridate. An extensive discussion of phosphorylation and phosphorylating agents can be found in Y. Mizuno, Studies in Org. Chem, 24, 171-175 (1986) and references cited therein.

30

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EXAMPLE 20

A culture of Rhizopus arrhizus MF 4974 from the Merck Culture Collection was inoculated in 50
5 milliliters of soy-glucose medium in a 250 milliliter flask and the flask incubated on a rotary shaker at 220 rpm at 27°C for 24 hours to obtain the first stage seed culture. 2.5 milliliters of this seed
10 culture was inoculated into each of 18 flasks containing 50 milliliters of soy-glucose medium which was also charged with Compound Z to a concentration of 5 µg/ml in 50 microliters of DMSO and the
15 resulting culture incubated on a rotary shaker at 220 rpm at 27°C for 18 hours.

Following incubation, each flask was harvested by centrifugation, the mycelium washed twice with sterile saline and resuspended in pH 7.0 phosphate buffer containing 1 percent glycerol. Compound Z was added to a final concentration of 58
20 µg/ml using 100 µl of (DMSO). The flasks were incubated on a rotary shaker at 220 rpm at 27°C for 48 hours.

At the end of this period, the contents of eighteen flasks (900 milliliters) were pooled and the
25 whole broth centrifuged. The mycelial cake was slurried with 100 milliliters of water, the pH adjusted to 3.5 and the slurry extracted twice with 100 milliliters of n-butanol. The supernatant was acidified to pH 3.5 and extracted twice with one-half
30 the volume of n-butanol. Each organic extract was assayed by HPLC. The assay condition was as follows:

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Column: "ZORBAX" (DuPont) C8 Rx 4.5 x 250 mm
Mobile Phase: Acetonitrile + 10mM aq. KH_2PO_4 ,
gradient 20% to 80% over 20 minutes
Temperature: 45°C
5 Flow rate: 1.5 ml/min.
Detection: 210 nm
Sample Vol: 50 μl

10 The extracts of the mycelium and the extracts of the supernatant were pooled and evaporated to dryness at 30°C at reduced pressure to obtain an oil as residue.

The oil was dissolved in the mobile phase of 40/60 acetonitrile/water and further purified using ZORBAX C8 (9.6 mm x 25 cm) semi-preparative column.
15 The column was developed at 7.05 ml/min. using 40 percent aqueous acetonitrile containing 0.1 percent trifluoroacetic acid (TFA) at 45°C. Fractions having a retention time of 12.8 minutes were pooled, and the solvent evaporated to obtain 12 mg of Compound III in
20 a yield which calculated to be 24%.

The product had the mass spectral data previously set forth.

A portion of the product was converted to the monopotassium salt. This was carried out by
25 dissolving the biophosphorylation product in 70 percent aqueous acetonitrile containing 10 mM KH_2PO_4 (pH 4.5). The mixture was subjected to reduced pressure to remove the acetonitrile and the aqueous residue loaded onto a water-equilibrated C-18 solid
30 phase extraction column. The column was washed with water and then eluted with 70 percent aqueous acetonitrile and the eluate freeze-dried for retention of the salt.

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EXAMPLE 21

5 Spores of Rhizopus arrhizus were obtained from oatmeal agar slants of MF 4974 maintained in the Merck Culture Collection and employed to prepare a spore suspension in water of about 7×10^9 spores per milliliter for use in the preparation of seed culture.

10 Seed flasks each containing 500 milliliters of soy glucose broth of the composition previously given, were inoculated with 1 milliliter of spore suspension and incubated on a rotary shaker (220 rpm) at 27°C for 24 hours.

15 Following incubation, the mycelia from each flask were harvested by filtering through a 10 micron nylon mesh and then resuspended in an equal volume of a 100 mM pH 6.3 phosphate buffer containing 3 percent glycerol and added to the broth. Compound Z, was added to a concentration of about 50 µg/ml in dimethylsulfoxide. The flasks were then incubated on
20 a rotary shaker at 220 rpm at 27°C for 24 hours.

 After completion of the incubation period, the contents of sixteen flasks totaling 8000 milliliters were pooled and filtered through a 10 micron nylon mesh. The mycelial cake was slurried
25 with 1000 milliliters of 50 percent aqueous methanol and filtered. The mycelial cake was again extracted with aqueous methanol and the two aqueous methanol filtrates combined and diluted with 2000 milliliters of water.

30 The resulting aqueous solution was applied to a 15 mm x 300 mm column packed to a 220 mm bed height with water equilibrated "DIANION" HP20 resin.

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The filtrate was pumped in a downflow mode at 15 ml/min. in 2000 ml aliquots. After loading, the column was washed with 500 milliliters of water and the desired phosphorylated product eluted with 500
5 milliliters of 20 percent aqueous acetonitrile. The remaining substrate and metabolite was eluted with 500 milliliters of 70 percent aqueous acetonitrile.

The diluted filtrates from the mycelial extracts were also pumped onto the column, the column
10 washed and then eluted and the eluate assayed by HPLC. The assay condition was as described in Example 20.

HPLC indicated the following mass balance for microbial phosphorylation and "DIAION" HP 20
15 isolation:

Substrate Charged:	402.5 mg
Metabolite & Substrate Recovered:	383.8 mg
Total Recovery:	95.4%
20 Compound III	234.9 mg
Bioconversion yield:	61.2%

The eluate was concentrated under reduced pressure and the residue dissolved in 40 percent
25 aqueous acetonitrile containing 10mM KH_2PO_4 to obtain a monopotassium salt. The aqueous solution was subjected to reduced pressure to remove the acetonitrile and the aqueous residue loaded onto a water-equilibrated C-18 solid phase extraction
30 column. The column was thereafter washed with water and then the monopotassium salt of Compound III eluted with 70 percent aqueous acetonitrile. The

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eluant was freeze dried to obtain the salt. The NMR spectrum of this salt is that previously detailed.

The salt is converted to the acid by careful acidification.

5

EXAMPLE 22

10 The following salts are prepared by reacting an appropriate phosphate salt and Compound III in the manner described in Example 21 and concentrating under vacuum.

	<u>Example No.</u>	<u>R</u>
15	IIIa	PO(OH)(ONa)
	IIIb	PO(OH)(OK)
	IIIc	PO(ONa) ₂
	IIId	PO(OLi)(OH)
	IIIe	PO(OH)(OMg) _½
20	IIIf	PO(OH)(ON(CH ₃) ₄)

EXAMPLE 23

25 1000 compressed tablets each containing 500 mg of Compound IIIA are prepared from the following formulation:

	<u>Compound</u>	<u>Grams</u>
30	Compound IIIA	500
	Starch	750
	Dibasic calcium phosphate hydrous	5000
	Calcium stearate	2.5

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The finely powdered ingredients are mixed well and granulated with 10% starch paste. The granulation is dried and compressed into tablets.

5

EXAMPLE 24

1000 hard gelatin capsules, each containing 500 mg of mono sodium salt of Compound III are prepared from the following formulation:

10

<u>Compound</u>	<u>Grams</u>
Compound III, mono sodium salt	500
Starch	750
15 Dibasic calcium phosphate hydrous	5000
Calcium stearate	2.5

A uniform mixture of the ingredients is prepared by blending and used to fill two-piece hard gelatin capsules.

20

250 ml of an injectable solution are prepared by conventional procedures having the following formulation:

25 Dextrose	12.5 g
Water	250 mL
Compound III, mono potassium salt	400 mg

30

The ingredients are blended and thereafter sterilized for use.

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EXAMPLE 25

An aerosol composition may be prepared
having the following formulation:

5

Per Canister

Compound IIIA	24 mg
Lecithin NF Liquid	
10 Concentrated	1.2 mg
Trichlorofluoromethane, NF	4.026 g
Dichlorodifluoromethane, NF	12.15 g

Preparation of Starting Materials

15

Compound Z, the starting material, was prepared by inoculating 54 milliliters of P34-2 medium of the following composition per liter: corn steep liquor, 5 g; D-mannitol 25 g; glucose monohydrate, 10 g; "PHARMAMEDIA," (nonhydrolyzed protein, Buckeye Oilseed Products, Memphis, Tenn.)
20 20 g KH_2PO_4 , 9 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 10 mg; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.25 mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 mg; H_3BO_3 , 0.56 mg; $(\text{NH}_4)_6 \text{Mo}_7\text{O}_{24} \cdot \text{H}_2\text{O}$, 0.19 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg, with frozen vials of Zalerion arboricola MF5533 ATCC
25 74030 and the inoculated medium incubated with shaking at 220 rpm at 25°C for four days. Twenty milliliters were used to inoculate four 2-liter flasks containing 500 milliliters of P34-2 medium and the inoculated medium incubated at 25°C for four days
30 at 220 rpm. The flask contents were pooled and used to inoculate into three fermenters each containing 180 liters of P34-2 medium and 2 ml/L of propylene

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glycol P-2000 (Dow Chemical) to reduce foaming and the inoculated medium cultivated for six days at 25°C, an air flow of 90 L/min. a pressure of 0.7 kg/cm² gauge, and an agitator speed of 200 rpm. A
5 25 liter sample of the resulting broth was then used to inoculate three fermenters each containing 475 liters of P34-2 medium containing 2 ml/L of P-2000 and cultivated for four days at 25°C, air flow of 250 L/min, pressure 0.7 kg/cm² gauge and 150 rpm.

10 425 liters of this seed broth was inoculated into each of three production fermenters containing 13,700 liters of TG106 medium of the following composition per liter: D-mannitol, 100 g; NZ-Amine type E (casein hydrolysate, Sheffield Products, Kraft
15 Inc.) 33 g; Fidco 8005 yeast extract (Difco), 10 g; (NH₄)₂SO₄, 5 g; KH₂PO₄, 9 g; P-2000, 2 ml, and the fermenters operated at a temperature of 27°C, air flow of 2500 liters/minute, a pressure of 0.7 kg/cm² gauge, and an agitator speed of 50 rpm. The pH was
20 allowed to decrease from 6.0 to 5.5 and then maintained at 5.5 ± 3. After about 2 1/2 weeks the broth was harvested for product isolation.

The broth from the foregoing cultivation was first extracted with an equal volume of methanol.
25 The methanol-broth was clarified using a liquid-solid separator (centrifuge) to obtain clarified liquid as first extract and solid. The extraction-clarification was repeated. The extracts were combined and the water content adjusted to about 50 percent. The
30 resulting solution was passed through a "DIAION" SP-207 adsorption column to adsorb Compound III and the column washed with aqueous methanol. Thereafter Compound III was recovered with 100 percent methanol.

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The water content of the methanol containing Compound III was adjusted to 50 percent and the aqueous methanol solution intimately mixed with an equal volume of 1:1 ethyl acetate/hexane and the two liquid phases thereafter separated. The aqueous methanol layer was passed through a column of "DIAION" SP-207, the column washed with aqueous methanol, and Compound III eluted with 100 percent methanol. The eluant was vacuum concentrated to a minimum volume and the solvent composition adjusted to about 75:20:5 ethyl acetate/methanol/water.

The feed thus prepared was passed through a silica gel column and Compound III eluted with 85:10:5 ethyl acetate/methanol/water. The fractions showing 85 percent or greater area purity by HPLC were combined, vacuum concentrated to remove ethyl acetate and the concentrate adjusted to 50 percent aqueous methanol, the latter passed through "DIAION" HP-20 in the manner previously described concentrated and Compound III precipitated with acetonitrile and recovered by vacuum filtration and then dried.

The starting material also may be prepared by methods described in copending applications Serial Nos. 47/492,025 and 47/492,026.

Z. arboricola MF5533 ATCC 74030 is disclosed and claimed in copending application Serial No. 630,457, filed December 19, 1990. Briefly, it may be obtained by (a) inoculating a frozen vegetative mycelium of Z. arboricola ATCC 20957 (disclosed and claimed in copending application Serial No. 492,024) into KF seed medium of: corn steep liquor, 5 g/l; tomato; tomato paste 40 g/l, oat flour 10 g/l;

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glucose 10 g/l; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 10 mg/l; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 10 mg/l; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.25 mg/l; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 mg/l; H_3BO_3 , 0.56 mg/l; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot \text{H}_2\text{O}$, 0.19 mg/l; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg/l; adding to the medium

5 N-methyl-N'-nitro-N-nitrosoguanidine (NTG) (b) cultivating, thereafter (c) plating a portion of the growth on potato dextrose agar and (d) incubating for 14 days at 25°C to obtain spores then (e) harvesting the spores, (f) diluting the spores with sterile

10 saline (g) plating on potato dextrose agar (h) incubating for 7 days for colony formation, (i) transferring separate colonies to slants of potato and (j) incubating for 14 days at 25°C.

15

EXAMPLE 26

Biotransformation Enzymology-Isolation Of Cell Free Activity

20

Spores of Rhizopus oryzae MF 4974, cultivated on oatmeal agar, were inoculated in a Soy-Glucose medium consisting of 20.0 g dextrose, 5.0 g Fidco yeast extract, 5.0 g NaCl, 5.0 g K_2HPO_4 in a liter of distilled water. The pH of the medium

25 was adjusted to 7.0 before autoclaving. The cultures were incubated at 27°C for 24 hours on a rotary shaker at 220 rpm. A fresh Soy-Glucose medium was inoculated with the 24-hour seed culture at a ratio of 5% (v/v) and the fermentations were continued in the manner

30 described above. The phosphorylating enzyme activity, as a function of time, was determined.

Cell free extracts were obtained by suspending the cells in a buffer containing 100 mM phosphate

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(pH 7.5) and 2 mM EDTA. The cells to buffer ratio was 2:1. Several procedures were then tried to break the cell membranes and release the cell contents, i.e., a high-pressure French press, sonication, grinding the frozen cells, and lysozyme treatment.

The enzyme reaction was carried out in 0.2 ml mixture containing 0.15 mM FK-520, 5 mM $MgCl_2$, 5 μM ATP and various amount of enzyme. The reaction mixture was incubated at different temperatures for 1 hour and terminated by the addition of 0.2 ml methanol. The resulting solution was subjected to HPLC analysis on Whatman Partisil 10 ODS-3 at 55°C. The column was developed at 1 ml/min with a linear gradient from 45% acetonitrile in 0.1% phosphoric acid to 80% in 0.1% phosphoric acid in 30 minutes. The retention time of phosphorylated FK-520 and FK-520 was 16.5 and 20 minutes, respectively. Protein concentration was determined by Pierce BCA protein assay reagent using BSA as standard.

In a search for phosphorylating enzyme in cell free extracts of Rhizopus oryzae MF 4974 we have found phosphorylating activity: 1) is highest in the extracts of cells isolated after 24 hours incubation in Soy-Glucose medium; 2) both sonication and maceration of the cells under liquid nitrogen can be used to break the cell membranes for liberating the enzyme in soluble form. The pH optimum is 7.5; 4) the temperature optimum is 37°C. 5) The phosphorylation of FK-520 was linear with respect to time and enzyme concentration for one hour and from 0.2 to 2.0 mg protein. The effect of the FK-520 concentration on the rate of phosphorylation have been studied using crude extract. The K_m value for the substrate

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(FK-520) was about 0.5 mM and the V_{max} was 2.1n mole/min/mg protein. To determine metal ion requirement the crude enzyme was dialyzed thoroughly against 20 mM Tris-HCl buffer and 2 mM EDTA. The results show that the phosphorylating activity requires Mg^{+2} or Ca^{+2} as cofactor.

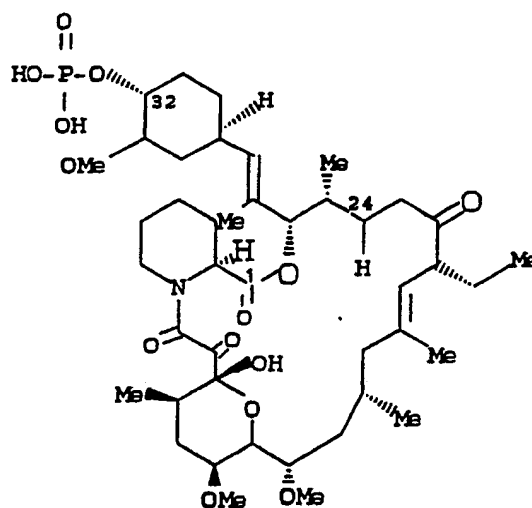
EXAMPLE 27

NMR analysis established that the biotransformation product obtained by incubating 24-deoxy FR-900520 with MF 4974 (*Rhizopus arrhizus*) is the 32 phosphorylated analog:

15

20

25



Contacting 24-deoxy FR 900520 made by the process described in Examples 28-29 with the *Rhizopus* microorganism as per the analogous procedure described for 24-oxy FR 900520, yields the 32-phosphorylated analog as determined by NMR spectroscopy.

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EXAMPLE 28

17-Ethyl-1-hydroxy-12-[2'-(3'',4''-dihydroxycyclo-
hexyl)-1'-methylvinyl]-23,25-dimethoxy-13,19,21,27-
5 tetramethyl-11,28-dioxa-4-azatricyclo[22.3.1.0^{4,9}]-
octacos-14.18-diene-2.3.10.16-tetraone

To a stirred solution of 31-desmethyl
FK-900,520, 17-ethyl-1,14-dihydroxy-12-[2'-(3'',4''-
dihydroxycyclohexyl)-1'-methylvinyl]-23,25-dimethoxy-
10 13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo-
[22.3.1.0^{4,9}]octacos-18-ene-2,3,10,16-tetraone
produced as described in EPO Publication. 0 349 061
(77 mg in 3 ml benzene) was added 6 mg of
p-toluenesulfonic acid and the mixture warmed to 60°C
15 in an oil bath. After 25 minutes, the reaction
mixture was cooled to room temperature, neutralized
by the addition of a saturated aqueous NaHCO₃
solution and extracted with ethyl acetate (3 times).
The combined organics were washed with saturated NaCl
20 solution, dried over Na₂SO₄ and purified by flash
chromatography (20% hexanes in ethyl acetate and 1%
MeOH) to yield 40 mg of the title compound.
MASS: (FAB) 782 (m + Na).
Partial ¹H NMR (200 MHz): δ 6.80 (dd, J₁ = 16 Hz, J₂
25 = 6 Hz), 6.16 (dd, J₁ = 16 Hz, J₂ = 1.5 Hz), 4.39
(broad d, J = 14 Hz), 4.26 (broad d, J = 5 Hz), 3.91
(dd, J₁ = 8.8 Hz, J₂ = 3 Hz).

30

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EXAMPLE 29

17-Ethyl-1-hydroxy-12-[2'-(3'',4''-dihydroxycyclo-
hexyl)-1'-methylvinyl]-23,25-dimethoxy-13,19,21,27-
5 tetramethyl-11,28-dioxa-4-azatricyclo[22.3.1.0^{4,9}]-
octacos-18-ene-2.3.10.16-tetraone

To a solution of 17-ethyl-1-hydroxy-12-
[2'-(3'',4''-dihydroxycyclohexyl)-1'-methylvinyl]-
23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-
10 azatricyclo[22.3.1.0^{4,9}]octacos-14,18-diene-2,3,10,16-
tetraone (40 mg in 1.5 ml ethyl acetate) was added 3
mg of 5% Rh/Carbon catalyst. The reaction flask was
fitted with a hydrogen balloon, evacuated and
recharged with hydrogen gas (3 times). After 45
15 minutes, the mixture was filtered over Celite,
concentrated and purified by flash chromatography
(CH₂Cl₂: MeOH: Hexane (10:1:2)). to yield 33 mg of
the title compound.

MASS: (FAB) 768 (m + Li).

20 Partial ¹H NMR (200 MHz): δ 4.55 (broad d, J = 5
Hz), 4.39 (broad d, J = 14 Hz), 3.86 (dd, J₁ = 8.8
Hz, J₂ = 3 Hz).

EXAMPLE 30

25

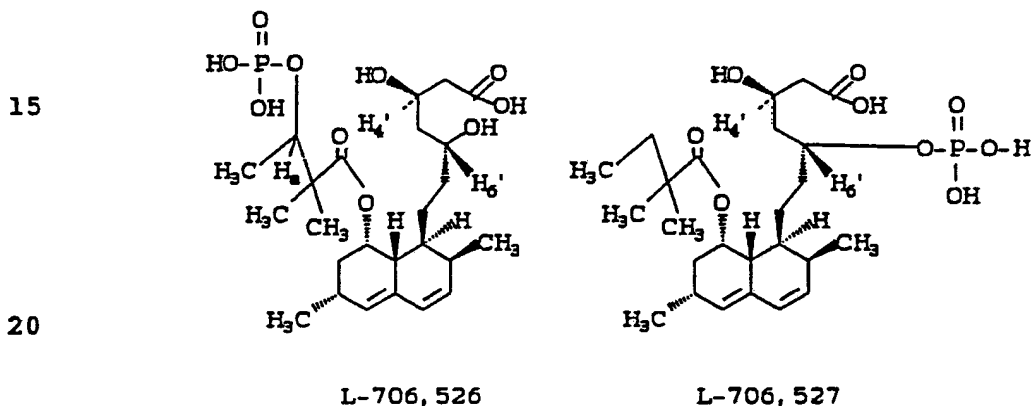
PHOSPHORYLATED ANTI-CHOLESTEROLEMIC
ISOLATES L-706.526 AND L-706.527

30 Contacting simvastatin, whose chemical name
is: 6(R)-[2-(8'(S)-2'',2''-dimethylbutanoyloxy-
2'(S),6'(R)-dimethyl-1',2',6',7',8',8'a (R)-
hexahydronaphthyl-1'(S)-ethyl]-4(R)-hydroxy-3,4,5,6-
tetrahydro-2H-pyran-2-one with the Rhizopus,

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microorganism under the analogous conditions described in Example 3 produced two phosphorylated compounds, L-706,546 and L-706,527. The preparation and properties of simvastatin are described in EP
5 Publication No. 0033538 to Merck & Co., Inc.

NMR analysis of the biotransformation products obtained by incubating the above compound simvastatin with Rhizopus arrhizus confirms their assigned structures as illustrated:
10



25 The compounds exhibit inhibition in the biosynthesis of cholesterol as also described for simvastatin in EP Publication No. 0 033 538.

Key features in the NMR of L-706,526 are the absence of a side chain methyl triplet and the
30 presence of a novel $\text{CH}_3\text{CH}-\text{O}-$ moiety with signals at 1.10 ppm (CH_3) and at 3.93 ppm ($\text{CH}-\text{O}$). Irradiating at 1.10 ppm collapsed the methine quintet to a

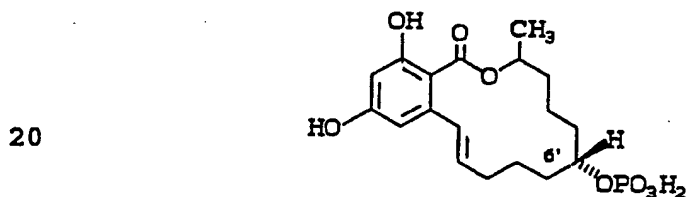
- 127 -

doublet. Since the methine proton in the proposed structure has no vicinal neighbor the appearance as a doublet implies coupling with an unseen hetero atom. i.e., the phosphorus.

5 Phosphorylation at the H-6' site^a is suggested by the absence of a typical H-6' signal at 3.69 ppm and the presence of a broad, featureless signal at 4.26 ppm which is assigned to a displaced H-6'. Both the magnitude of the displacement and the
10 loss of detail are reasonable consequences of phosphorylation.

EXAMPLE 31

15 Phosphorylation of Zearalenone



Chemical structure of
 α -zearalenol-6'-phosphate

25 Incubation of zearalenone available from Sigma with resting cells of Rhizopus arrhizus MF 4974 for 24 hours results in two biotransformation products. They have been identified as α -zearalenol and α -zearalenol-6'-phosphate. The fermentation,
30 isolation, purification and enzymatic hydrolysis are described below.

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FERMENTATION

5 One Ml of a Rhizopus arrhizus (MF 4974) spore suspension, harvested from oatmeal agar, was incubated into 500 mL Soy-Glucose medium contained in a 2 liter Erlenmeyer flask and shaken at 27°C on a rotary shaker (220 rpm) for 24 hours.

10 Following incubation, each flask was harvested by filtration washed once with water, then resuspended in 500 mL of mM PO_4 buffer (pH 7.0) containing 4% glycerol. Zearalenone was added to a final
15 concentration of .05 mg/mL, using 0.8 mL DMSO. The charged flasks were incubated on a rotary shaker (220 rpm) at 27°C for 24 hours. Following incubation, the whole
20 broth was worked up as described below.

	<u>Media</u>	<u>Soy Glucose Medium</u>	<u>g/L</u>
		Dextrose	20.0
		Soy Meal	5.0
25		Fido yeast extract	5.0
		NaCl	5.0
		K_2HPO_4	5.0
30		Adjust pH to 7.0	

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ISOLATION AND PURIFICATION

The whole broth (1000 mL) was diluted with an equal volume of methanol, than centrifuged. The mycelial cake was discarded. The clear filtrate was applied to a 15mm X 30 cm column packed with HP 20 (Mitsubishi Chemical, 220 mm bed height water equilibrated). The filtrate was pumped onto the column in the downflow mode at 15 mL/min. After loading, the column was washed with 500 mL water. Metabolites or substrates were not detected in the spent broth or wash. The column was eluted with a step gradient of methanol:water. The most polar metabolite eluted between 10% and 30% methanol. The second metabolite and substrate eluted in the column wash (100% methanol). Appropriate fractions were combined and evaporated to dryness under reduced pressure at 30°C to yield yellow oils that was subjected to further purification by preparative HPLC. Preparative HPLC was carried out on Whatman Magnum 9 Partisil 10 ODS-3 column (C₁₈, 9.8 mm ID x 25cm) at room temperature and monitored at 237 nm. The column was developed at 4 mL/min with linear gradient from 20% acetonitrile in 0.1%phosphoric acid to 80% acetonitrile in 0.1% phosphoric acid in 40 minutes.

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The compounds were collected during repeated injections of the above described extract. Fractions of retention times 17.5 and 20.1 minutes were collected, pooled, diluted with three volumes of water and desalted using a Speed C₁₈ column (Applied Separations) to yield 7 mg of 6-phosphate ester and 5 mg of α -zearalenol, respectively.

ENZYMATIC HYDROLYSIS

To prove that the 6'- α -hydroxyl group is phosphorylated, enzymatic hydrolysis was examined using various sulfatases and alkaline phosphatases obtained from Sigma. In summary, a specific amount of enzyme was added to one mL of 100mM phosphate buffer containing ~50 ug/mL of zearalenol phosphate ester. The buffer solution was incubated at 37°C for 4 hours. After incubation, 300 uL was removed and analysed by HPLC, using a Whatman Partisil 10 ODS-3 column (C₁₈ 10 μ m, 4.6 mm ID x 25 cm) at room temperature and monitored at 237 nm. The column was developed at 1 mL/min with a linear gradient gradient from 20% acetonitrile in 0.1% phosphoric acid to 80% acetonitrile in 0.1% phosphoric acid in 20 minutes. Under these conditions, α -zearalenol-6'-phosphate ester and α -zearalenol have retention times of 13.93 and 18.17 minutes, respectively.

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The results of enzymatic hydrolysis are summarized below:

	<u>ENZYME</u>	<u>pH</u>	<u>UNITS</u>	<u>ACTIVITY</u>
5	P-4653			
	Alk. Phosphatase	7.0	1.0	100% α -OH @ 1hr
	P-5521			
	Alk. Phosphatase	7.0	100.0	100% α -OH @ 1hr
10	S-8629			
	Sulfatase	5.0	10.0	0% α -OH @ 1hr
	S-1629			
	Sulfatase	7.0	1.0	100% α -OH @ 1hr
	P-8639			
15	Alk. Phosphatase	7.0	10.0	6% α -OH @ 1hr
	P-1391			
	Alk. Phosphatase	7.0	10.0	100% α -OH @ 1hr
	S-9751			
20	Sulfatase	5.0	100.0	52% α -OH @ 1hr

CHARACTERIZATION

The compounds were identified by MS and NMR and confirmed by enzymatic hydrolysis as α -zearalenol-6'-phosphate and α -zearalenol. Also, the presence of phosphorus was confirmed by an ICP elemental analysis.

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WHAT IS CLAIMED IS:

1. A process for producing a biophosphorylated hydroxyl containing organic compound, wherein
5 said hydroxyl group is phosphate reactive, comprising the step of contacting a strain of Rhizopus oryzae microorganism together with the hydroxy containing organic compound in an aqueous medium containing a carbon nutrient at ambient temperature for a suffi-
10 cient time to produce the biophosphorylated hydroxyl containing organic compound.
2. The process of Claim 1 wherein said
15 microorganism comprises resting cells of Rhizopus oryzae in a phosphate buffer containing glycerol as a carbon nutrient.
3. The process of Claim 1 wherein said
20 Rhizopus oryzae is cultured with said hydroxyl containing organic compound under submerged aerobic fermentation conditions in an aqueous carbohydrate medium containing a nitrogen nutrient at a pH below about 8.0 at ambient temperature.
- 25 4. The process of claim 1 wherein said
Rhizopus oryzae strain is ATCC No. 11145.

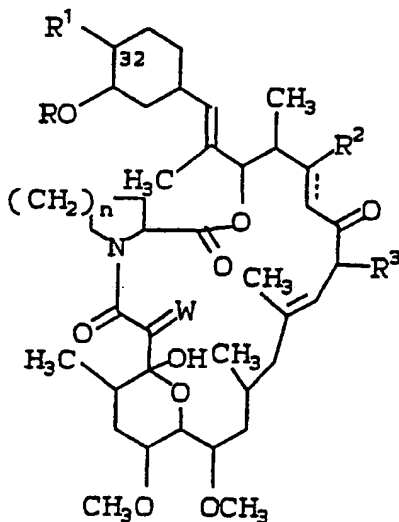
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5. A process for producing an immunosuppressant, identified as a C-32 phosphorylated derivative of an FK-506 type macrolide comprising the step of contacting a strain of a Rhizopus oryzae microorganism together with a FK-506 type macrolide, containing a free C-32 hydroxy group, in an aqueous medium containing a carbon nutrient at ambient temperature for a sufficient time to produce the C-32 phosphorylated FK-506 type macrolide immunosuppressant.

6. A compound being the C-32 phosphorylated derivative of an FK-506 type macrolide.

7. The compound of Claim 1 of the formula:



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wherein:

R is H, C₁-C₄ alkyl,

R¹ is H₂PO₄,

R² is hydrogen, hydroxy or lower alkanoyloxy,

5 R³ is methyl, ethyl, propyl or allyl,

n is an integer of 1 or 2, and the symbol of a
line and dotted line is a single bond or a
double bond,

and a pharmaceutically acceptable salt thereof.

10

8. The compound of Claim 7 being the C-32
phosphorylated derivative of FK-506, FK-520, FK-523,
FK-525, C-31 desmethyl FK-506, C-31 desmethyl FK-520.

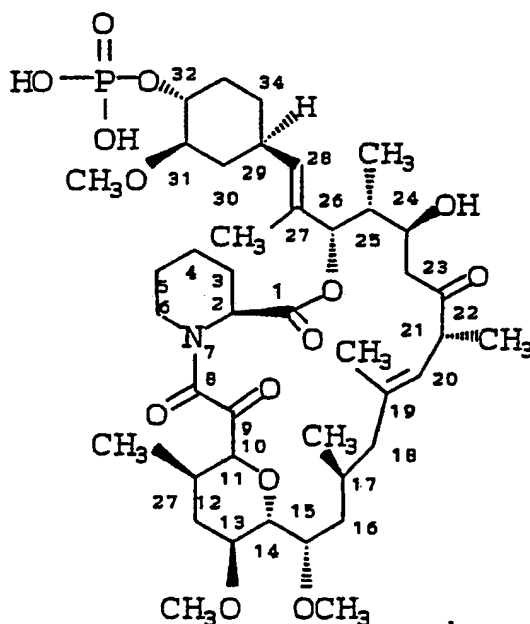
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9. The compound of Claim 7 being:

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10. The compound of Claim 7 being:

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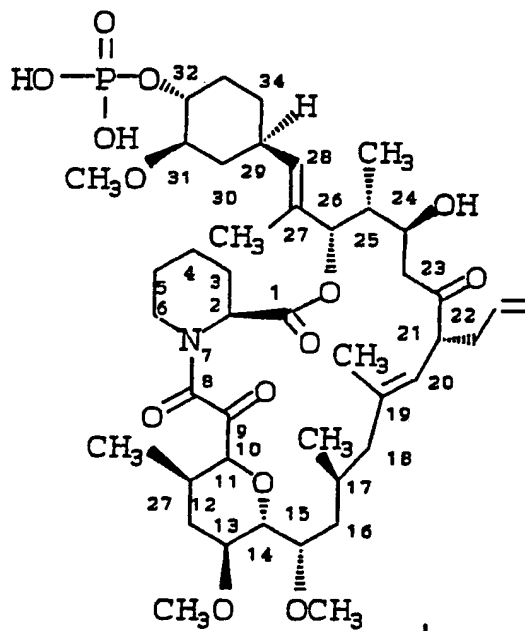
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11. The compound of Claim 7 being:

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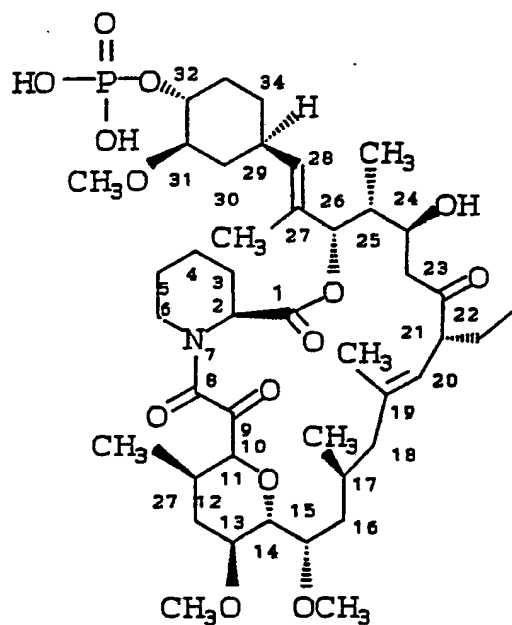
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12. The compound of Claim 7 being:

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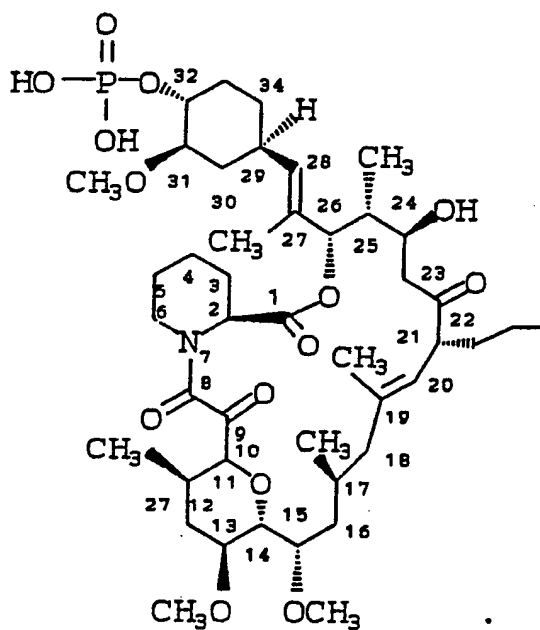
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13. The compound of Claim 7 being:

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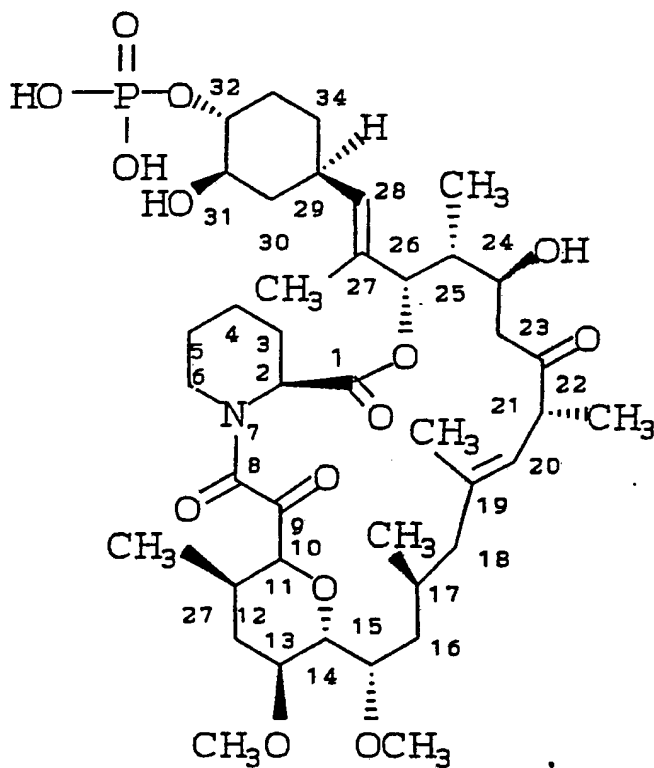
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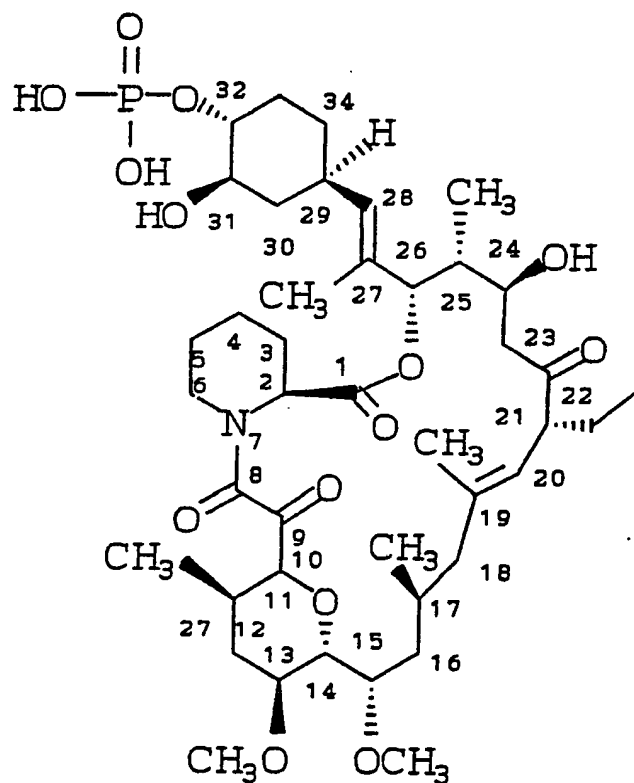
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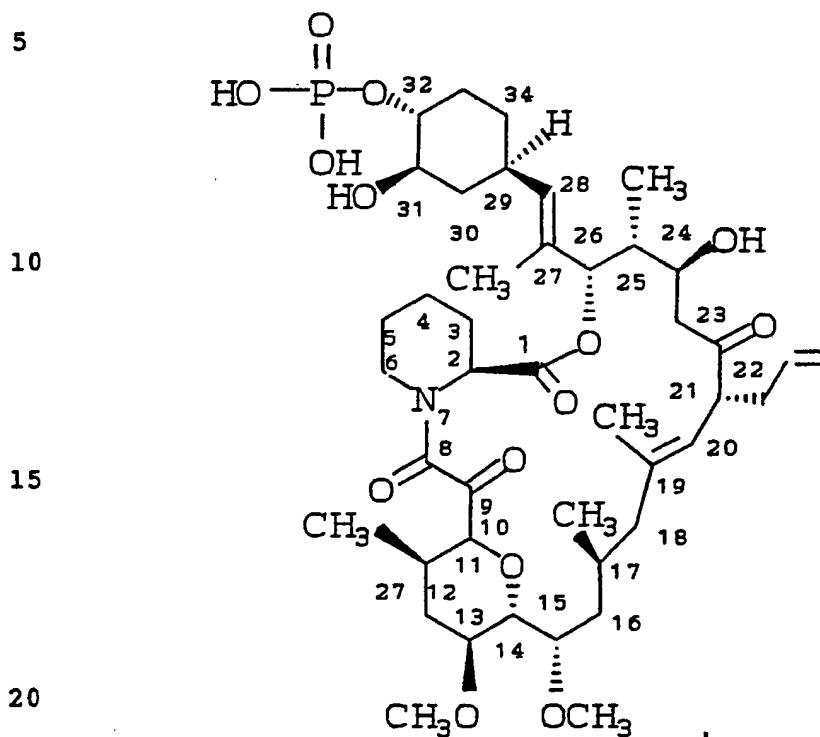
- 139 -

14. The compound of Claim 7 being:



- 140 -

15. The compound of Claim 7 being:



16. A pharmaceutical composition for the
 25 treatment of immunoregulatory disorders or diseases
 comprising a pharmaceutical carrier and a
 therapeutically effective amount of compound of Claim
 7.

30 17. A pharmaceutical composition for the
 topical treatment of inflammatory and
 hyperproliferative skin diseases and or cutaneous
 manifestations of immunologically-mediated illnesses

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comprising a pharmaceutical carrier and a therapeutically effective amount of compound of Claim 7.

5 18. A method for the treatment of immunoregulatory disorders or diseases comprising the administration to a mammalian species in need of such treatment an effective of a compound of Claim 7.

10 19. A process for producing an immunosuppressant, identified as a phosphorylated rapamycin-type macrolide comprising the step of contacting a strain of a Rhizopus oryzae
15 microorganism together with rapamycin macrolide in an aqueous medium containing a carbon nutrient at ambient temperature for a sufficient time to produce the C-43 phosphorylated rapamycin macrolide immunosuppressant.

20 20. The process of Claim 19 wherein said microorganism are resting cells of Rhizopus oryzae in a phosphate buffer containing glycerol as a carbon nutrient.

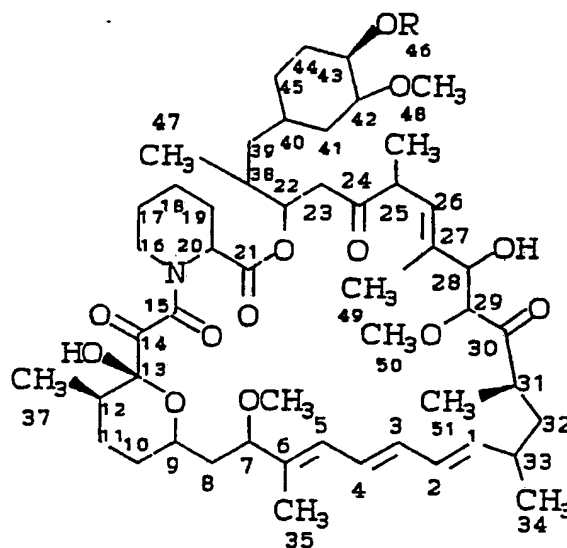
25 21. The process of Claim 19 wherein the resulting aqueous medium containing said phosphorylated macrolide exhibits positive inhibition of T-Cell activation.

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22. The process of Claim 19 wherein said Rhizopus oryzae is cultured with said rapamycin macrolide under submerged aerobic fermentation conditions in an aqueous carbohydrate medium containing a nitrogen nutrient at a pH below about 8.0 at ambient temperature.

23. A phosphorylated macrolide of the structural formula:



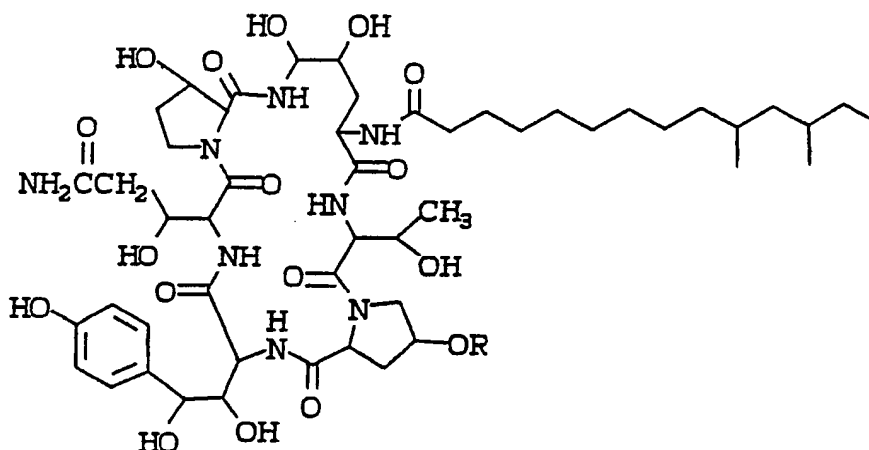
where R= $\begin{array}{c} \text{O} \\ || \\ \text{[HO]2-P-} \end{array}$

24. A phosphorylated macrolide exhibiting a characteristic proton NMR spectrum as illustrated in Figure 3 and exhibiting positive inhibition of T-cell proliferation, reversible by recombinant human IL-2, in a T-cell proliferation assay.

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25. A phosphorylated macrolide having the structural formula as illustrated in Figure 4.

26. A method for producing a phosphorylated cyclic lipopeptide compound having the formula



wherein R is $\text{-}\overset{\text{O}}{\underset{\text{||}}{\text{P}}}(\text{OH})_2$ which comprises cultivating
 25 Rhizopus arrhizus ATCC 11145 in a nutrient medium
 containing a compound having the formula:

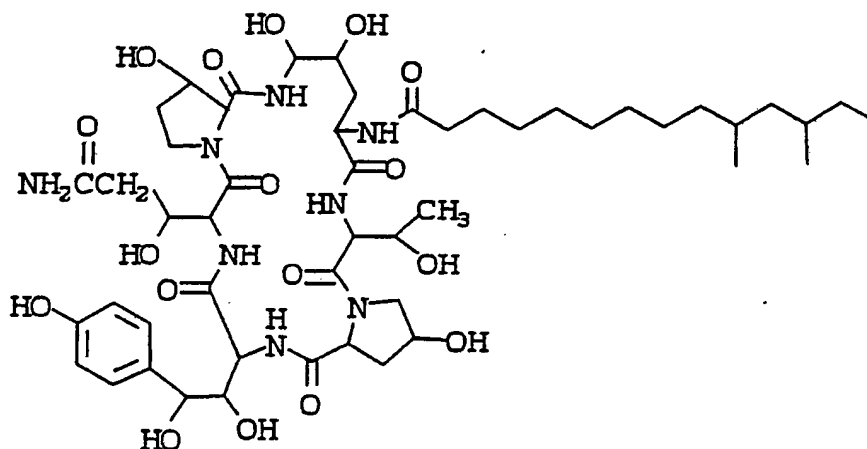
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and a phosphate salt, said medium maintained in a pH range of from about 6.0 to 6.3.

20

27. A method according to Claim 26 in which the phosphate salt is in an amount of at least 10 percent by weight of the solid components.

25

28. A method according to Claim 26 in which the medium of the following composition in grams/liter: glucose 20.0; soya meal 5.0; Fidco yeast extract 5.0; sodium chloride 5.0; and potassium hydrogen phosphate 5.0; at pH 5.

30

29. A method according to Claim 26 in which the cultivation is carried out in the temperature range of from about 15°C to about 30°C with agitation in the range of 220 rpm to 400 rpm for a period of from 24 hours to about two days.

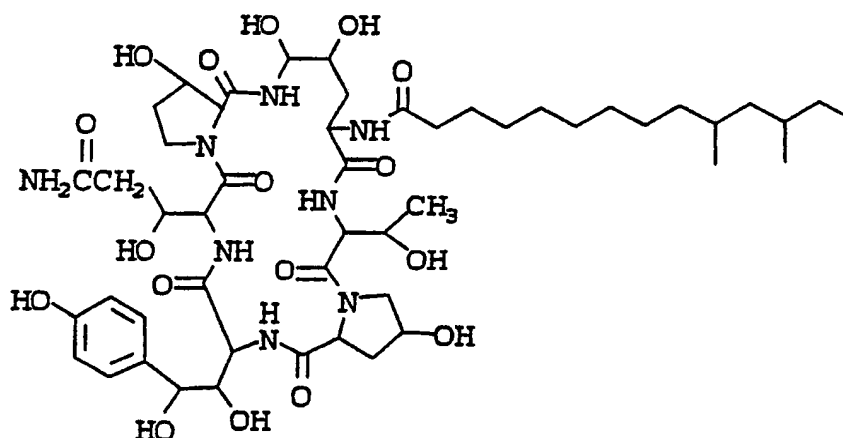
- 145 -

30. A method for selectively
phosphorylating a compound having the formula

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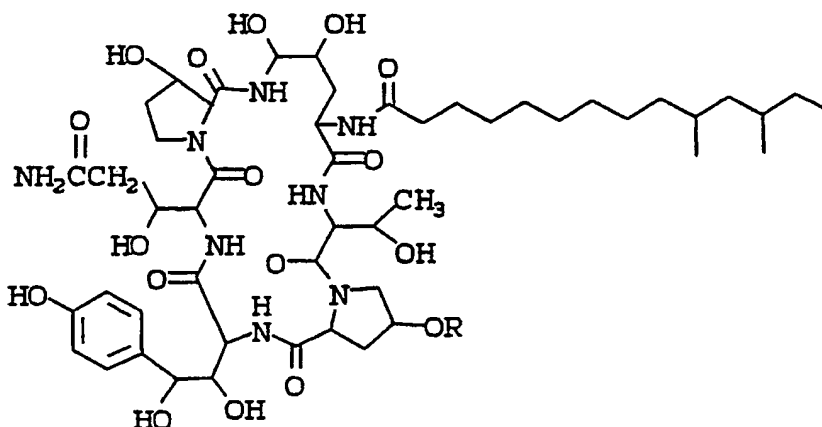
at the 4-hydroxyproline hydroxyl which comprises
cultivating Rhizopus arrhizus ATCC 11145 in a soy
glucose medium containing said compound in an amount
of 40-60 µg/ml, wherein said soy glucose medium is of
the following composition in grams/liter: glucose
20.0; soya meal 5.0; Fidco yeast extract 5.0; sodium
chloride 5.0; and potassium hydrogen phosphate 5.0;
at pH 5.

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31. A phosphorylated cyclic lipopeptide compound obtained by biophosphorylating with Rhizopus arrhizus ATCC 11145, a cyclic lipopeptide related to echinocandins and having a peptide skeleton bearing several hydroxy groups wherein in said phosphorylated cyclic lipopeptide, the phosphate group is attached to the hydroxy group of the 4-hydroxyproline component of the lipopeptide.

32. A phosphorylated cyclic lipopeptide compound having the formula:



wherein R is $\text{-}\overset{\text{O}}{\parallel}\text{P}(\text{OH})_2$ or a cation salt thereof.

33. A compound according to Claim 32 wherein R is $\text{-}\overset{\text{O}}{\parallel}\text{P}(\text{OH})_2$.

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34. A compound according to Claim 32 wherein

R is $\overset{\text{O}}{\underset{\parallel}{\text{P}}}(\text{OH})(\text{OK})$.

5

35. A compound according to Claim 32 wherein

R is $\overset{\text{O}}{\underset{\parallel}{\text{P}}}(\text{OH})(\text{ONa})$.

10

36. A composition comprising the compound of Claim 32 in admixture with a pharmaceutically acceptable carrier.

15

37. A method for controlling the growth of fungi comprising administering an antifungally effective amount of the compound of Claim 32.

20

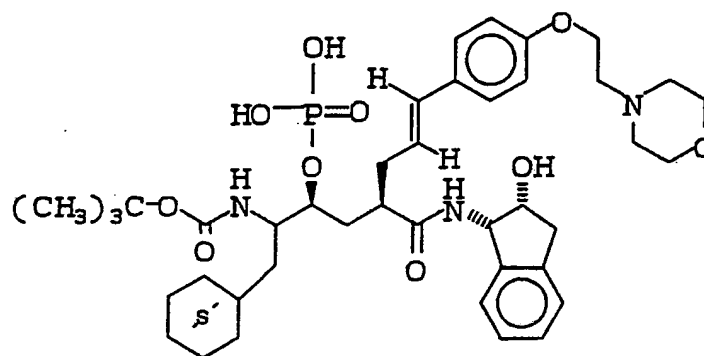
38. A method for the treatment of or for the prevention of Pneumocystis carinii infections in mammals which comprises administering to mammals an anti-infective or therapeutic amount of the compound of Claim 32.

25

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39. A compound of the Formula:



or pharmaceutically acceptable salt, hydrate, or
ester thereof.

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40. A method of preparing the compound of Claim 39, comprising the steps of

- 5 (a) providing a quantity of N-(2(R)-hydroxy-1(S)-indanyl)-5(S)-(1,1-dimethylethoxy-carbonylamino)-4(S)-hydroxy-2(R)-[(4-(2-(4-morpholino)ethoxy)phenyl)prop-2-en-1-yl]-6-cyclohexyl-hexanamide;
- 10 (2) incubating the compound of step (a) in a microbial culture of Rhizopus arrhizus MF4974; and
- (3) isolating the compound of Claim 39.

15 41. A pharmaceutical composition useful for inhibiting HIV protease, comprising an effective amount of a compound of Claim 39, and a pharmaceutically acceptable carrier.

20 42. A pharmaceutical composition useful for preventing or treating infection of HIV or for treating AIDS or ARC, comprising an effective amount of a compound of Claim 39, and a pharmaceutically acceptable carrier.

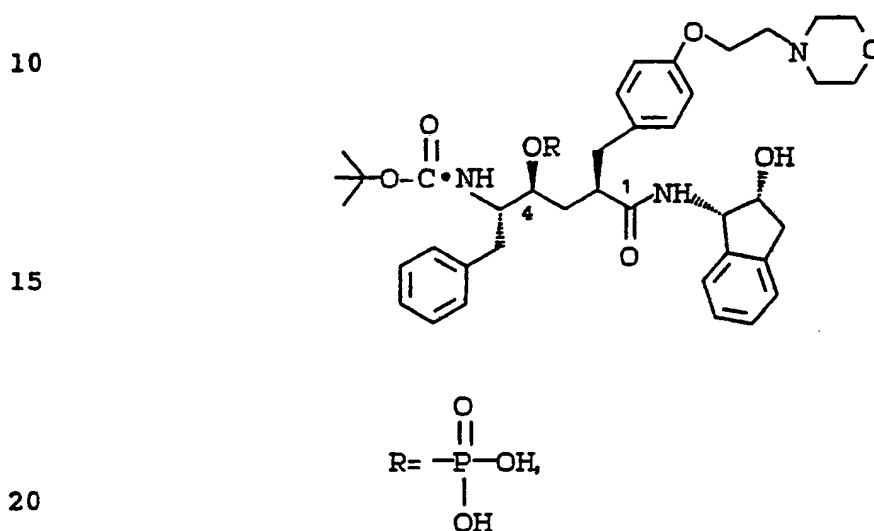
25 43. A method of inhibiting HIV protease, comprising administering to a suitable mammal in need of such treatment an effective amount of a compound of Claim 39.

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44. A method of preventing infection of HIV,
or of treating infection by HIV or of treating AIDS
or ARC, comprising administering to a suitable
mammal in need of such treatment an effective amount
5 of a compound of Claim 39.

45. A compound of the Formula:



or pharmaceutically acceptable salt, hydrate, or
ester thereof.

25 46. A method of preparing the compound of
Claim 45, comprising the steps of

30 (1) providing a quantity of N-(2(R)-hydroxy-
1(S)-indanyl)-5(S)-(1,1-dimethylethoxy-
carbonylamino)-4(S)-hydroxy-6-phenyl-
2(R)-[(4-(2-(4-morpholinyl)ethoxy)-
phenyl)methyl]-hexanamide;

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- (2) incubation the compound of step (1) in
a microbial culture of Rhizopus
arrhizus MF4974; and
(3) isolating the compound of Claim 45.

5

47. A pharmaceutical composition useful for
inhibiting HIV protease, comprising an effective
amount of a compound of Claim 45, and a
pharmaceutically acceptable carrier.

10

48. A pharmaceutical composition useful for
preventing or treating infection of HIV or for
treating AIDS or ARC, comprising an effective amount
of a compound of Claim 45, and a pharmaceutically
acceptable carrier.

15

49. A method of inhibiting HIV protease,
comprising administering to a mammal an effective
amount of a compound of Claim 45.

20

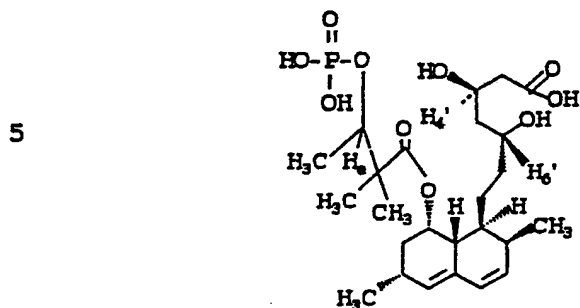
50. A method of preventing infection of HIV,
or of treating infection by HIV or of treating AIDS
or ARC, comprising administering to a mammal an
effective amount of a compound of Claim 45.

25

51. A phosphorylated simvastatin derivative
of the following structure:

30

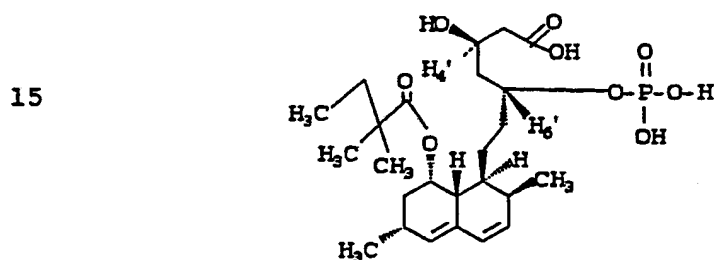
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L-706,526

or

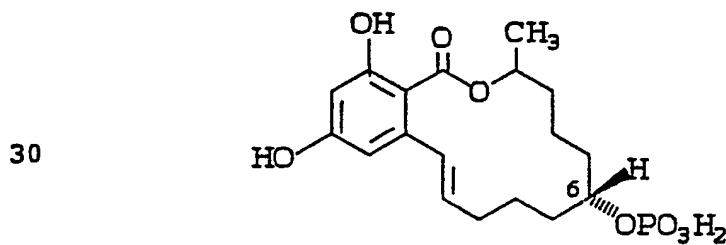


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L-706,527

52. A phosphorylated zearalenol compound of the following structure:

25



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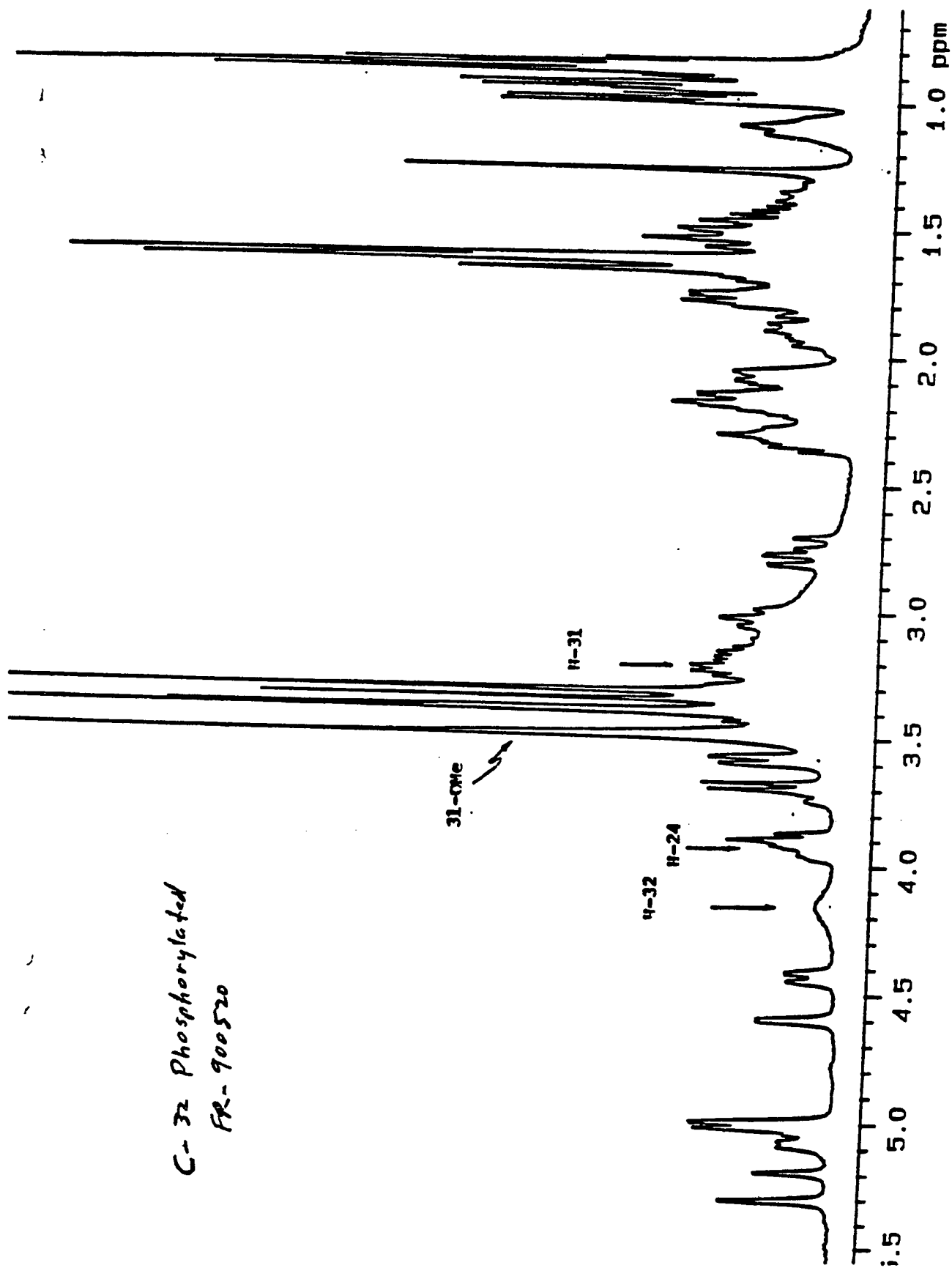


Figure 1

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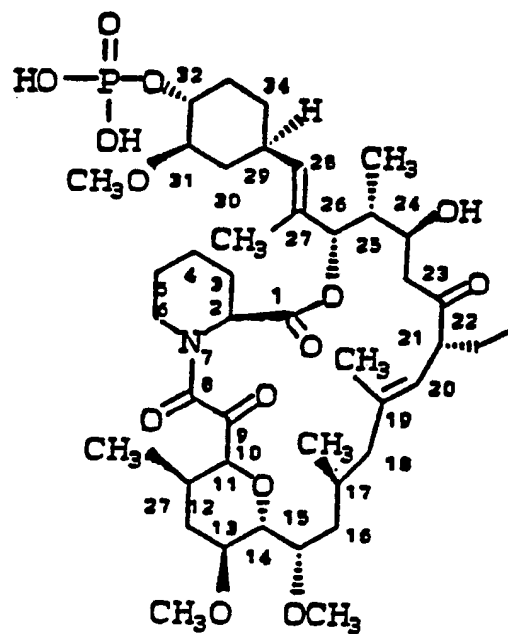


FIGURE 2

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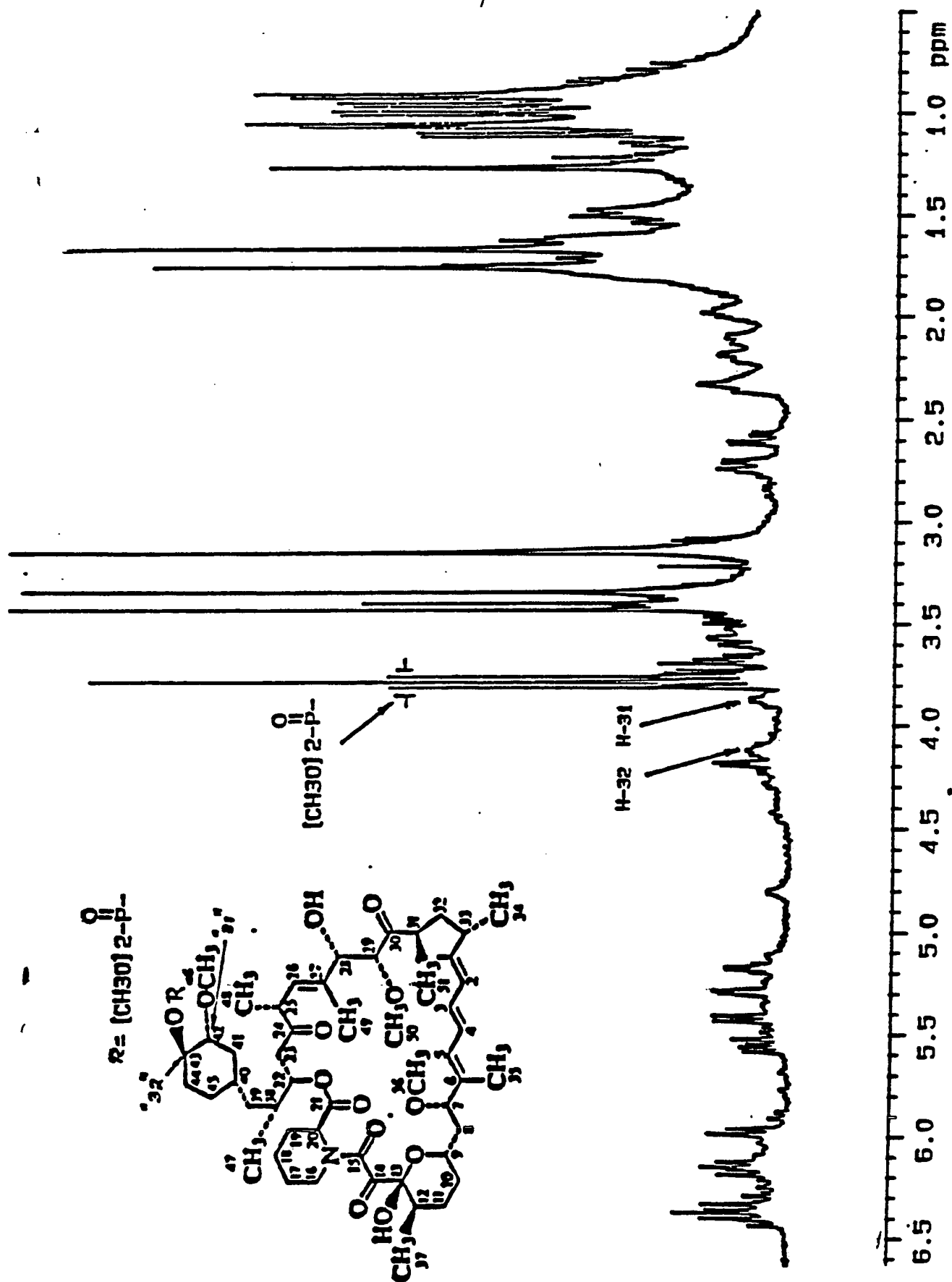


Figure 3

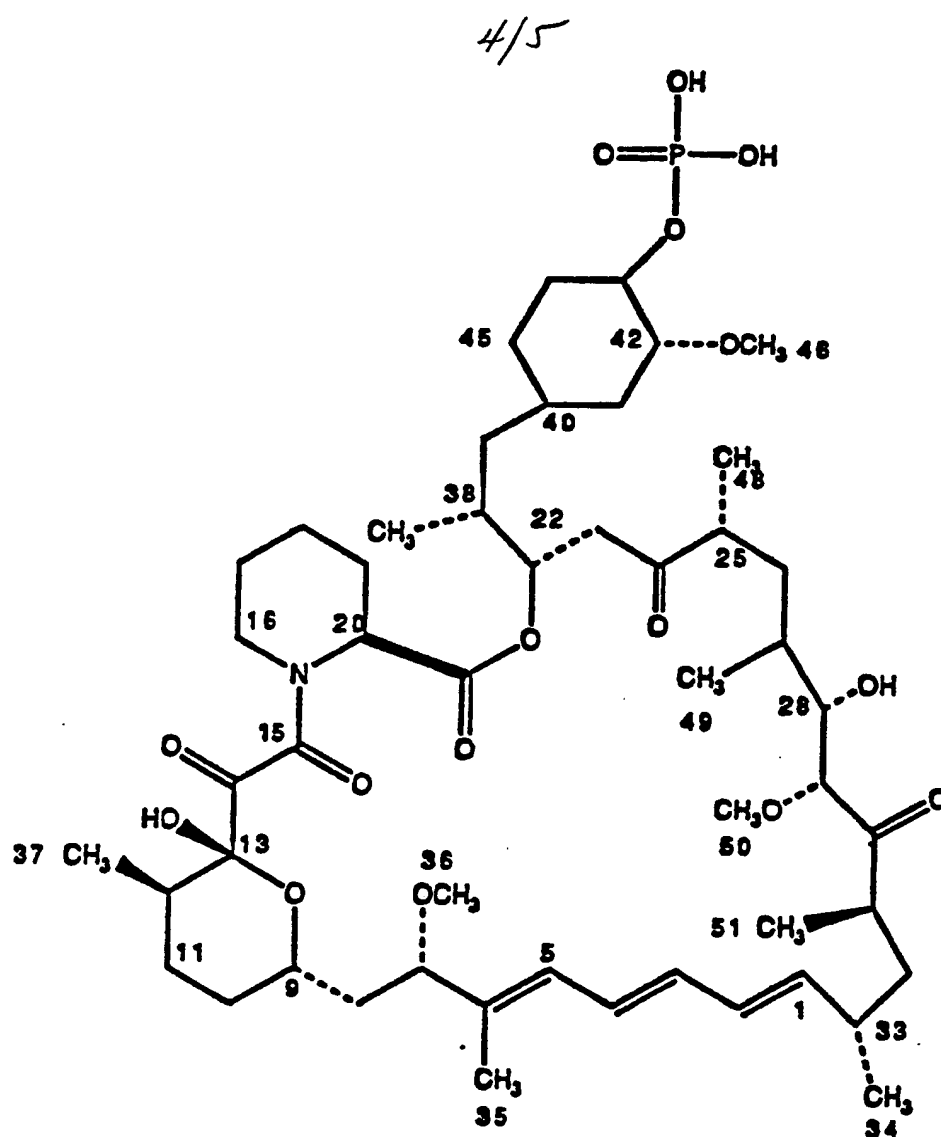


Figure 4

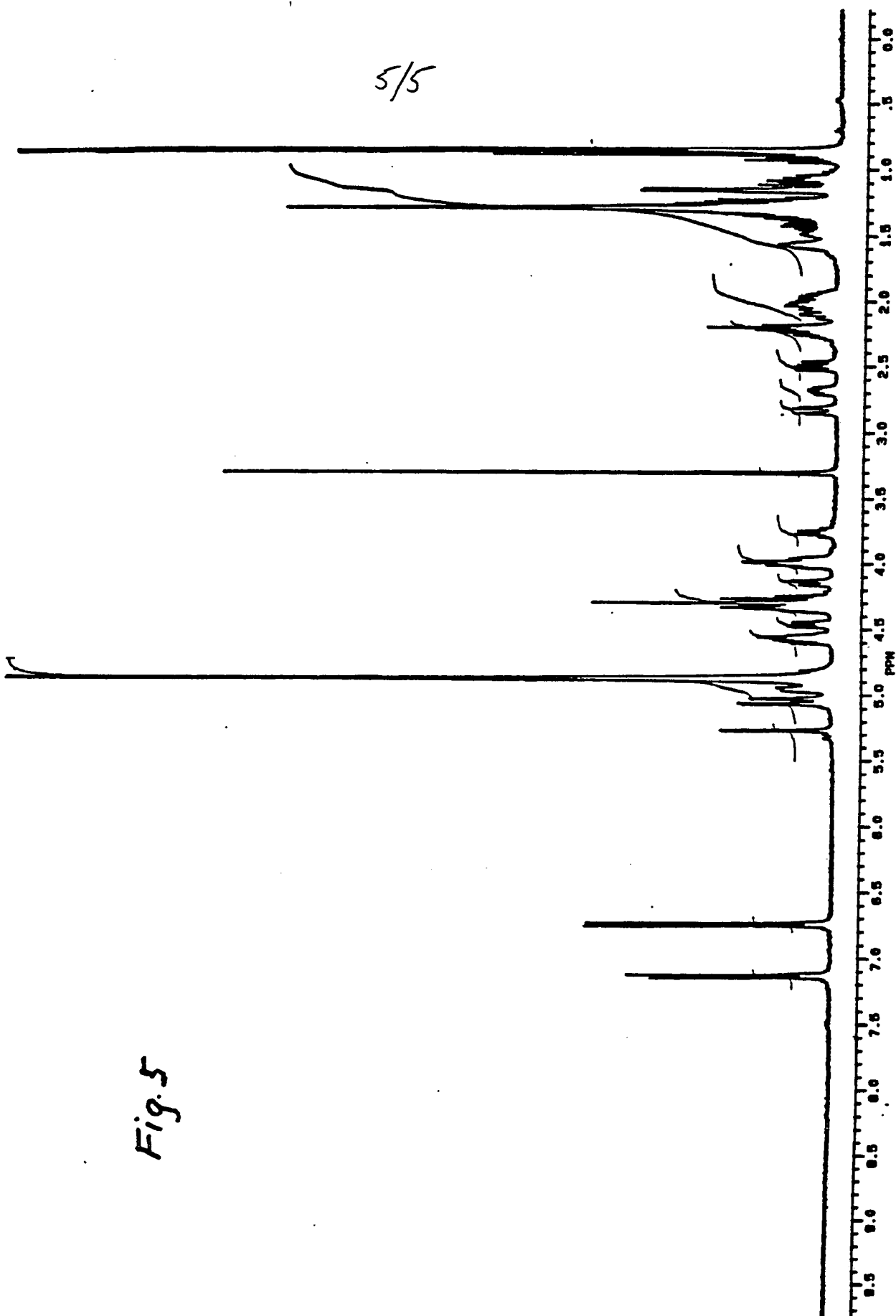


Fig. 5

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